DEPARTMENT OF CHEMISTRY

WILLIAM MARSH

RICE UNIVERSITY

STUDIES OF MUSTARD OIL GLUCOSIDES (II)

by

Martin G. Ettlinger and Charlyne P. Thompson

Final Report

Contract DA 19-129-QM-1689

Project 7-99-01-001, Simplified Food Logistics

AD_____ Accession No._____ W. M. Rice University, Houston, Texas STUDIES OF MUSTARD OIL GLUCOSIDES (II) - M. G. Ettlinger and C. P. Thompson Final Report, 1 Nov 62, i+106 pp-8 tables, 3 figs (Contract DA 19-129-QM-1689) QM Proj 7-99-01-001, Simplified Food Logistics, Unclassified Report Part I is an analytical catalogue of the mustard oils furnished by seeds of Brassica vegetables, including cabbages, turnips, mustards and their allies. Part II describes the acceleration by Vitamin C of the enzymatic release of mustard oils from their glucosides and covers enzyme purification, the effects of chemical modification of the vitamin and the reaction mechanism.	UNCLASSIFIED 1. Mustard Glucosides-Studies (II) 2. Contract DA 19-129-QM-1689	AD_____ Accession No._____ W. M. Rice University, Houston, Texas STUDIES OF MUSTARD OIL GLUCOSIDES (II) - M. G. Ettlinger and C. P. Thompson Final Report, 1 Nov 62, i+106 pp-8 tables, 3 figs (Contract DA 19-129-QM-1689) QM Proj 7-99-01-001, Simplified Food Logistics, Unclassified Report Part I is an analytical catalogue of the mustard oils furnished by seeds of Brassica vegetables, including cabbages, turnips, mustards and their allies. Part II describes the acceleration by Vitamin C of the enzymatic release of mustard oils from their glucosides and covers enzyme purification, the effects of chemical modification of the vitamin and the reaction mechanism.	UNCLASSIFIED 1. Mustard Glucosides-Studies (II) 2. Contract DA 19-129-QM-1689
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Commanding General
Quartermaster Research and
Engineering Command
Natick, Massachusetts

Attention: Dr. L. Long, Jr., Project Officer

Sir:

I have the honor to present a final report of research on mustard oil glucosides as under Contract DA19-129-QM-1689, Project 7-99-01-001, Simplified Food Logistics.

Some explanation may be given of the form and time of this report. The results of the preceding, three-year contract (DA19-129-QM-1059, Project 7-84-06-032) under the same title were embodied in a document delivered to you in June, 1961, the dissertation of Dr. George Dateo. The investigation there recorded, principally synthetic, formed a whole and included the fruits of the later contract up to May 1, 1961. Subsequent work until November 20, 1961, was devoted toward the completion of analytical and enzymatic studies begun years before under sponsorship of the Robert A. Welch Foundation. It was felt that a literal transcript of the parts done under the contract with your laboratories would be fragmentary and not fully intelligible. Therefore it was decided to take the occasion to assemble the conclusions of the work extending from previous years to the summer of 1962, lastly with support from the Aldrich Chemical Company and the R. T. French Company, into finished units. It is hoped that loss of individual detail will be more than offset by increased scope and ultimate value.

The survey of the chemical composition of Brassica vegetable seeds might well be deemed too monstrously extensive for so slight a topic, virtually home economics. The outcome does offer reassurance of the consistency of our horticultural categories and may suggest future possibilities and limitations of chemical taxonomy. The study of the coenzymatic function of ascorbic acid in mustard has been published in abbreviation (G. P. Dateo, Jr., B. W. Harrison, T. J. Mabry and C. P. Thompson, Proc. Natl. Acad. Sci. U. S., 47, 1875 (1961); 48, 305 (1962)) and is more clearly an essay in pure science. The present account derives from an original long version written in the summer of 1961, augmented by later results. Both sections of the report, at all events, have made an end.

The hard, scrupulous and long continued efforts of (Mrs.) Charlyne Thompson, research assistant, deserve grateful recognition.

Yours respectfully,

Martin Ettlinger

Martin Ettlinger

October 31, 1962

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Part I

Mustard Oils of Cultivated *Brassica* Seeds

Introduction

The mustard family, Cruciferae, contains important food plants, most of which are classified in the genus Brassica. The species and varieties of Brassica include the hot mustards, brown and black, whose seeds are used in condiments; several kinds of rape, whose seed furnishes fatty oil and animal feed; and a goodly number of vegetables eaten by man as shoots, leaves or roots. Familiar sorts are mustard greens, turnips, rutabagas, cabbages, kales, collard greens, Brussels sprouts, cauliflower and broccoli. The members of Brassica, like others of their family, yield isothiocyanates or mustard oils, which are major flavoring constituents and may be transformed to compounds that as thyroid inhibitors could affect public health. The investigation presented was a chromatographic survey to ascertain the types of isothiocyanates occurring in Brassica frequently cultivated in the United States and correlate the plants with their chemical composition. The part chosen for analysis was the ripe seed because it is easily stored and generally produces the desired compounds in high concentration. Mention will be made of the known similarities and differences between the isothiocyanates of the seed and the edible portions of the vegetables.

Botanical Prologue

Although the broad taxonomic outlines of cultivated Brassica have been apparent for a long while, the detailed arrangement offered difficult problems to the classical, morphological approach. Liberty Hyde Bailey, dean of American horticultural botanists, exclaimed "These brassicas are the most baffling plants I have ever studied" and again "The Brassica group is indeed perplexing, excepting Rubus [the blackberries] the most bewildering I have

attempted. These plants were not closely examined by botanists until they had become widely dispersed over the earth and had assumed unidentifiable disguises."[1] The modern classification, which affords a satisfactory general framework for the chemical results, can be based in the first instance on cytogenetic considerations. As the plants were arranged by Musil[2] for the U. S. Department of Agriculture, from data reviewed fully by Yarnell,[3] it is possible to recognize three elementary cultivated species, B. nigra, with haploid chromosome number n of 8, B. oleracea (n = 9) and B. campestris (n = 10), and two principal amphiploid hybrids, B. juncea (n = 18) from nigra and campestris, and B. napus (n = 19) out of campestris and oleracea. Further segregation, particularly of B. campestris into Western and Oriental (B. chinensis) species, has been discussed but is not clearly related to chemical composition. So defined, B. nigra is black mustard, B. oleracea the cabbages and allies, among them kales, collards, Brussels sprouts, kohlrabi, cauliflower and broccoli. B. campestris includes turnips in both root and foliage sorts, the European and Indian oilseed turnip-rapes, and several Far Eastern forms, of which Chinese cabbages and tendergreen are most familiar. B. juncea comprises brown, Oriental and foliage mustards, and B. napus rape, rutabagas and rape-kales, notably the Siberian type.

The genus Sinapis, closely related to Brassica, contains two well-known species, S. arvensis (n = 9), the charlock, and S. alba (n = 12), the cultivated white or yellow mustard. These species have often been merged[2] into Brassica, preferably under the respective names B. kaber (DC.) Wheeler and B. hirta Moench. However, besides the morphological distinction upheld in Schulz's standard classification[4,5] and the failure[3] of S. arvensis and B.

oleracea, albeit of the same chromosome number, to yield normal hybrids, the sharp chemical difference of the two genera so far as known renders the separation useful to maintain.

The place of the cultivated Brassica in a standard general classification[5] of Cruciferae is worth notice. Schulz allotted the 350 genera of the family among 19 tribes, one of which was Brassiceae, containing 52 genera. The Brassiceae were divided into 7 subtribes, of which the first two, Brassicinae[4] (11 genera) and Raphaninae[4] (18 genera), included all the cultivated food plants. Brassicinae contained Brassica, Sinapis and the roquette Eruca, Raphaninae the radish Raphanus, as well as Crambe (sea kale). Each genus of course possessed wild besides cultivated species. Brassica, the largest, comprised a total of perhaps 35 species out of more than 150 in the two subtribes.

A perspective view of the mustard oils of cultivated Brassica ought to include a considerable number of related plants. An American worker is poorly situated to examine the group of wild Brassicinae and Raphaninae, which is entirely native to the Old World, being most concentrated in the Mediterranean region. In the present study, samples in the subtribes were obtained from European botanical gardens. Nevertheless, not only was the collection small and random, but the identities of the seed, without facilities to grow the plants and compare them with herbarium specimens, remained uncertain.[6] The generosity of the donating gardens is respectfully acknowledged, but they are not compelled by the financial need to maintain reputation that enforces the warranties of commercial seed houses. The results must be accordingly interpreted.

Chemical History

The natural isothiocyanates have lately been reviewed by Kjaer,[6] whose summary may be consulted for all but the most recent references. The mustard oils themselves are secondary products in the sense that they are not appreciably present free in intact plant material but are formed after maceration by enzymatic hydrolysis of glucosidic precursors as shown in the equation.

$$R\text{-}C(=NOSO_3^-)SC_6H_{11}O_5 + H_2O \xrightarrow[(L\text{-Ascorbate})]{\text{Enzyme}} RNCS + C_6H_{12}O_6 + H^+ + SO_4^=$$

The reaction resembles the liberation of prussic acid from cyanogenetic glycosides. It is however convenient to speak of isothiocyanates simply as plant constituents for the following discussion.

Table I shows the mustard oils of known concern here. The first eleven listed form a group related by homology, gain or loss of methanethiol, and oxidation-reduction. The last three, aromatic isothiocyanates are of specialized occurrence. Nasturtiin is common in roots of crucifers,[6] including Brassica and turnip[7] for example, but seems to be at most a minor constituent in seed of the two subtribes considered. 3-Indolylmethyl isothiocyanate[8] is found in fresh plants of Brassica and Raphanus but not in the seed. 4-Hydroxybenzyl isothiocyanate is characteristic of Sinapis seed but scarcely detectable in relatives outside that genus.

Three of the mustard oils in Table I may be important to public health because they yield antithyroid substances.[9] 3-Indolylmethyl and 4-hydroxybenzyl isothiocyanates readily evolve thiocyanate ion, and 2-hydroxy-3-butenyl isothiocyanate spontaneously cyclizes to 5-vinyl-2-thiooxazolidone or goitrin.

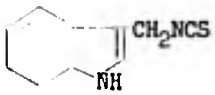
The mustard oils in Brassica and related plants have been studied frequently,[6] although many investigations were limited to the volatile oils, excluding the sulfoxides, sulfones and unstable aromatic compounds. B. nigra seed is the classic source of allyl isothiocyanate. In B. oleracea, a highly polymorphous species, allyl isothiocyanate is also prominent, except for broccoli,[10] but the other components are notable and varied. KJaer's early results[10] show as

Table I

Mustard Oils of Brassicinae and Raphaninae

Structure	Name
$H_2C{=}CHCH_2NCS$	Allyl isothiocyanate
$H_2C{=}CH(CH_2)_2NCS$	3-Butenyl isothiocyanate
$H_2C{=}CH(CH_2)_3NCS$	4-Pentenyl isothiocyanate
$[H_2C{=}CHCHOHCH_2NCS]$	Goitrin ((-)5-vinyl-2-thioxazolidone, from 2-hydroxy-3-butenyl isothiocyanate)
$H_2C{=}CH\underset{O}{\overset{CH_2\text{-}NH}{CS}}$	
$CH_3S(CH_2)_3NCS$	Ibervirin (3-methylthiopropyl isothiocyanate)
$CH_3S(CH_2)_4NCS$	Erucin (4-methylthiobutyl isothiocyanate)
$CH_3SO(CH_2)_3NCS$	Iberin (3-methylsulfinylpropyl isothiocyanate)
$CH_3SO(CH_2)_4NCS$	Sulforaphane (4-methylsulfinylbutyl isothiocyanate)

Table I (continued)

$CH_3SOCH{=}CH(CH_2)_2NCS$	Sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate)
$CH_3SO(CH_2)_5NCS$	Alyssin (5-methylsulfinylpentyl isothiocyanate)
$CH_3SO_2(CH_2)_3NCS$	Cheirolin (3-methylsulfonylpropyl isothiocyanate)
$C_6H_5(CH_2)_2NCS$	Nasturtiin (2-phenylethyl isothiocyanate)
	3-Indolylmethyl isothiocyanate
	4-Hydroxybenzyl isothiocyanate

minor constituents of the seed 3-butenyl isothiocyanate and what can now be recognized as ibervirin. Work at the U. S. Army Quartermaster Laboratories[11] on white cabbage heads found likewise allyl and 3-butenyl isothiocyanates and ibervirin. Studying non-volatile oils of fresh parts, Procházka[12] obtained good evidence of iberin and a lesser amount of sulforaphane. "Caulorapin" from kohlrabi seed[13] probably was a similar mixture including sulforaphane. Finally, goitrin is present in seed and tops of B. oleracea.

In Brassica campestris, allyl isothiocyanate recedes to a trace. Compounds with a longer carbon chain, 3-butenyl isothiocyanate and goitrin, are dominant in the seed and goitrin occurs also in turnip root. The two amphiploids based on B. campestris usually differ sharply from each other chemically. Seed of American or European B. juncea contains, like the B. nigra parent, a great preponderance of allyl isothiocyanate.[14] Recent workers have found, however, confirming an old report, that Indian B. juncea seed may contain 3-butenyl isothiocyanate, alone or mixed with its lower homologue.[15] B. napus as rape seed furnishes, like B. campestris, 3-butenyl isothiocyanate and goitrin, accompanied by the higher 4-pentenyl isothiocyanate. Rutabaga seed principally contains goitrin, also conspicuous in the edible root.

The occurrence of 4-hydroxybenzyl isothiocyanate in seed of Sinapis alba and S. arvensis[16] has already been indicated. Among other Brassicinae, Eruca sativa (roquette) and Diplotaxis tenuifolia yield erucin, D. erucoides furnishes allyl isothiocyanate and Erucastrum gallicum allyl with a little 3-butenyl mustard oil. In Raphaninae, Raphanus sativus (radish) seed is the standard source of sulforaphene, with in one variety a trace of allyl

isothiocyanate. Crambe maritima contains allyl isothiocyanate, Rapistrum perenne the 3-butenyl homologue, and Rapistrum rugosum cheirolin.

Analytical Methods

Plants containing mustard oils may be analyzed qualitatively in several ways.[6] The glucosides can be extracted with hot solvent to denature protein and chromatographed on paper. On the other hand, the enzymatic liberation of isothiocyanates can be allowed to proceed and the products separated by vapor chromatography if volatile, on paper if not. Finally, the isothiocyanates can be treated with ammonia ($RNCS + NH_3 \rightarrow RNHCSNH_2$) and the resulting thioureas identified by paper chromatography and estimated quantitatively by ultraviolet absorption. The last procedure, originally applied by Kjaer and co-workers[17] to volatile mustard oils from numerous crucifers, was used in the work to be discussed as an adaptation that arose from a study by Hodgkins[18] and was further developed by Miss Barbara Harrison with support from a Welch Foundation grant. The enzymatic reaction is performed in presence of a large excess of immiscible solvent to extract isothiocyanates and thiooxazolidones as formed. The extract is contaminated mainly by fatty oil, but the seed was not defatted routinely because of the trouble and possibility of loss. A special analysis is required for 4-hydroxybenzyl isothiocyanate, which is not converted to a thiourea by ammonia but decomposed to thiocyanate ion as by alkali.

For determination of 4-hydroxybenzyl isothiocyanate in Sinapis, ground seed (1/2 - 1 g.) was macerated three hours at room temperature in 5 ml. of water and 100 ml. of ether (free of alcohol and peroxides). The ether was decanted and, after washes with several small portions of water, extracted with 0.1 N sodium hydroxide. The alkali was let stand a quarter hour, neutralized with nitric acid and washed thoroughly with ether. An aliquot

was diluted to 10 ml. with solution containing 0.1 g. of ferric nitrate crystals and 0.05 ml. of concentrated nitric acid, and the absorbancy was measured at 470 millimicrons against a blank of the reagent. In 1-cm. cells, one microequivalent of thiocyanate ion (corresponding to 0.165 mg. of 4-hydroxybenzyl isothiocyanate) in the aliquot gave an absorbancy of roughly 0.35.

For more general applications, the maceration of seed was carried out as described, perhaps with addition of a little crude glucosinolase[19] and 5-10 mg. of sodium ascorbate. A sample (10-20%) of the ethereal solution was withdrawn to be tested for 4-hydroxybenzyl mustard oil and the remainder was divided into two equal portions. One half was evaporated to dryness, the residue (containing thiooxazolidones and non-volatile isothiocyanates) was dissolved in ethanol, the ultraviolet absorption of a suitable dilution was measured at 220, 240 and 260 millimicrons, and the solution was evaporated and examined by paper chromatography. The other half was treated with 10-25 ml. of ethanol and 2-5 ml. of concentrated aqueous ammonia, let stand four hours at room temperature or overnight at 5°, and evaporated to dryness. The residue, of thiooxazolidones and thioureas, was analyzed by spectrophotometry and chromatography like the first.

Thioureas and thiooxazolidones in ethanol have an intense absorption maximum near 240 mμ, in comparison with which the absorption of isothiocyanates[18] is negligible. The absorbancy at 240 mμ of the thiooxazolidone sample was corrected by a well-known method[17] for background assumed linear with wave length: the average of the absorbancies at 220 and 260 mμ was subtracted from the central value, and the difference if positive was

multiplied by the factor (K) that for the pure compound would restore the observed figure at 240 mμ. For goitrin, K was 1.2 and ε(240) 15000. The difference spectrum between ammonia-treated and untreated samples was assumed to be thiourea absorption. The thiourea spectrum could be corrected as before, but the calculated result at 240 mμ was not used if greater than the observed difference. The needed constants were: for allylthiourea,[17] K 1.45, ε 12600; for 3-butenylthiourea[17] and the derivatives of erucin, iberin, sulforaphane, cheirolin and alyssin, K 1.33, ε 12150; for sulforaphene thiourea, estimated K 1.6, ε 12500. The total isothiocyanate contents were reckoned in terms of one of the major components found by chromatography. The range in molecular weight, from allyl isothiocyanate to alyssin, is just under a factor of two.

Paper chromatography was conducted by ascent in a manner generally similar to that of Kjaer and Rubinstein.[20] The outer vessel was a cylindrical glass battery jar, 9 in. diameter by 1 ft. high, sealed with a glass plate. The developing organic solvent, saturated with water, was contained in a round crystallizing dish, 15 cm. diameter by 7-1/2 cm. deep. The conjugate aqueous phase was placed in the outer jar, and large sheets of filter paper dipping into each phase promoted saturation of the vapor space. The chromatographic strips, 3-1/2 by 11 in., were suspended magnetically[21] and equilibrated with the vapor for 15 hours with chloroform and 2-5 hours with other solvents before contact with the liquid. The developed chromatograms were sprayed with Grote's reagent[22] and steamed gently to produce blue, blue-green or violet spots, which were marked for intensity and location, from thioureas and thiooxazolidones. Each strip

bore two samples beside the standard phenylthiourea, compared to which the position of other compounds was expressed as R_{Ph}, the fraction of distance travelled.[20]

The primary solvent was wet chloroform.[20] Hydrophilic thioureas, with R_{Ph} less than 0.1 in chloroform, were resolved by a mixture (upper phase) of 3 parts of n-butanol, 1 part of toluene and 1 of water. Alyssin thiourea and methylthiourea were discriminated with use of 1 part of butanol, 3 parts of toluene and 2 of water. To assist identification of compounds with R_{Ph} above 0.6 in chloroform, the following solvents were employed: 1 part of butanol, 10 parts of toluene and 2 of water; 5 parts of toluene, 2 of acetic acid and 4 of water; 1 part of ethanol, 5 parts of benzene and 2 of water. The R_{Ph}-values for mustard oil derivatives of interest were determined with seeds of known composition or pure compounds, the indispensable need for most of which was generously met by Dr. Anders Kjaer. The numerical results were subject to considerable variation (± 0.05) from temperature fluctuations and obscure causes, so that continued judicious use of standards was necessary to interpret the chromatograms. Values of R_{Ph} for substances thought likely to be encountered in Brassicinae-Raphaninae, plus a few alternates of special interest, in 3:1 butanol-toluene were: iberin thiourea 0.35, thiourea 0.4, cheirolin and sulforaphane thioureas 0.42, 4-methylsulfonylbutylthiourea and sulforaphene thiourea 0.5, alyssin thiourea 0.6 (0.15 in 1:3 butanol-toluene), methylthiourea 0.6 (0.23 in 1:3 butanol-toluene), ethylthiourea 0.8 (0.6 in 1:3 mixture), allylthiourea 0.9 (0.75 in 1:3 mixture). Data for more hydrophobic compounds are given in Table II.

The translation of spots on chromatograms into a chemical composition depends on many ambiguities. It can usually be recognized which spots correspond to the principal substances, but the relative rank of lesser components, based only on visual estimates of color intensity, is highly uncertain. Frequently a minor ingredient will appear to be present with some solvents but not with others. Since the total quantity of material placed on a chromatogram is ordinarily governed by the desire not to overload spots of the major compounds, the lower limit of sensitivity is based on relative, not absolute, amount. In other words, 1/2 g./kg. of butenyl isothiocyanate would be more readily observed in mixture with 2 rather than 10 g./kg. of the allyl analogue. That a

Table II

Values of R_{Ph}

Compound	Solvent			
	Chloroform	Benzene-ethanol	Toluene-acetic acid	Butanol-toluene (1:10)
Allylthiourea	0.25	0.25	0.2	0.45
Isopropylthiourea	0.4			
3-Methoxycarbonyl-propylthiourea	0.5			
3-Butenylthiourea	0.6	0.6	0.45	0.85
Ibervirin thiourea	0.75	0.65	0.47	0.9
2-Butylthiourea	0.75	0.75	0.7	1.0
Benzylthiourea	0.92	1.13	0.85	1.2
4-Pentenylthiourea	0.92	1.03	0.9	1.15
Erucin thiourea	0.98	1.05	0.8	1.15
Nasturtiin thiourea	1.1	1.45	1.25	1.25
Berteroin thiourea (5-Methylthiopentyl-thiourea)	1.1	1.5	1.3	1.33
Goitrin	1.1	1.35	1.15	1.1
5,5-Dimethyl-2-thiooxazolidone	1.25			

substance is not listed for a seed implies no proof of complete absence, but merely that not enough was present to be certainly detected. Another complication arises from unknown compounds that turn pink with Grote's reagent and may mask the blue spots of interest. The sulforaphane region in 3:1 butanol-toluene chromatograms of B. campestris, for example, may sometimes be thus affected.

Qualitative identifications from chromatograms must also be hedged with caution. Allyl and butenyl isothiocyanates do indeed seem, when abundant, to be fairly unmistakeable, but for others the possibility of confusion with known or unknown compounds giving a derivative of similar mobility rarely is negligible. The assignments in this work depend critically on analogy to the isolations reported by others from the two subtribes investigated. All the substances to be listed have been covered by such isolation except alyssin, which is structurally related to 4-pentenyl isothiocyanate. Cheirolin and sulforaphane are inseparable by the technique used, and the spot was attributed to sulforaphane except for Rapistrum rugosum, whose composition was already known. The choice of sulforaphane might be justified in some instances by the conspicuous presence of the corresponding sulfide, erucin. Furthermore sulforaphane, but not cheirolin, has been reported from Brassica, and no evidence appeared of 4-methylsulfonylbutyl isothiocyanate, the homologue of cheirolin. Possible compounds with R_{Ph} above 1.0 in chloroform, aside from goitrin, were but sketchily examined. An immense number of natural thioureas of that class is known, and no satisfactory chromatographic scheme to distinguish all is available. Several unknown minor compounds have been passed over without specific notice, but

mention may be made of a violet spot at R_{Ph} 0.1 in 3:1 butanol-toluene chromatograms of numerous Brassica, recorded as "(+ hydrophilic oil)." The spot, although widespread in occurrence, sometimes did not appear on re-examination with fresh extracts and may represent a decomposition product.

The experimental conclusions are assembled in Table III, in fashion hoped to be straightforward. The species of Brassica are ordered alphabetically, followed by the samples of uncertain position, other genera of Brassicinae, and Raphaninae. The predominant isothiocyanate is underlined if necessary for distinction, and the contents of total isothiocyanates (exclusive of thiooxazolidones) and of goitrin are given separately. Commercial seed sources included Houston retail stores; the Burpee Company, of Philadelphia and Clinton, Iowa; the Ferry-Morse Co., of Detroit; Vaughan's, of Chicago; Hastings, of Atlanta; Reuter, of New Orleans; Sordillo's, which supplied many Italian imports, of Boston; Pearce Seed Co., Moorestown, New Jersey; and Clyde Robin, Carmel Valley, California. Specimens were kindly provided also by the R. T. French Company, Rochester, New York; Drs. H. S. Irwin and O. S. Fearing, then at the University of Texas; R. Bacigalupi, Berkeley, Calif.; and botanic gardens of Cologne, Paris, Rome and Vienna.

Table III

Mustard Oil Compositions of Seeds

of *Brassicinae* and *Raphaninae*

Brassica

Brassica barrelieri

Brassica barrelieri (L.) Janka [Brassica sabularia Brot., from Paris]	Goitrin	3-1/2 g./kg.
var. oxyrrhina (Coss.) Schulz [Brassica oxyrrhina Coss., from Paris]	Goitrin	1-1/2 g./kg.

Brassica campestris

Turnips

Amber Globe	3-Butenyl isothiocyanate	3 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	+ hydrophilic oils	
	+ goitrin	1-1/2 g./kg.
Early Purple Top Milan	3-Butenyl isothiocyanate	2 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	(+ hydrophilic oil)	
	+ goitrin	2-1/2 . /kg.
Early Purple Top Strap-leaved	3-Butenyl isothiocyanate	2 g. as butenyl/kg.
	+ 4-pentenyl (unusually much)	
	+ alyssin + hydrophilic oils	
	+ goitrin	3 g. as goitrin/kg.
	(+ hydrophobic thiooxazolidone)	
Early White Flat Dutch	3-Butenyl isothiocyanate	1 g. as butenyl/kg.
	+ 4-pentenyl + allyl + alyssin	
	+ sulforaphane (+ hydrophilic oil)	
	+ goitrin	3 g./kg.
Early White Milan	3-Butenyl isothiocyanate	2 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	+ goitrin	1 g./kg.

(contd.)

Brassica campestris

Turnips
(contd.)

European White Egg	3-Butenyl isothiocyanate	4 g. as butenyl/kg.
	+ 4-pentenyl + hydrophilic oils	
	+ goitrin	(0.3 g./kg.)
Flat White	3-Butenyl isothiocyanate	2 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	(+ hydrophilic oil)	
	+ goitrin	3 g./kg.
Golden Ball	3-Butenyl isothiocyanate	3-1/2 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	+ goitrin	1 g./kg.
Long White Cowhorn	3-Butenyl isothiocyanate	1-1/2 g. as butenyl/kg.
	+ 4-pentenyl	
	+ alyssin (+ hydrophilic oil)	
	+ goitrin	1 g./kg.
Pomeranian	3-Butenyl isothiocyanate	3 g. as butenyl/kg.
	+ 4-pentenyl + (minor) allyl	
	+ alyssin + hydrophilic oils	
	+ goitrin	1 g./kg.
Purple Top	3-Butenyl isothiocyanate	2 g. as butenyl/kg.
	+ alyssin (+ hydrophilic oil)	
	+ goitrin	3 g./kg.

(contd.)

Brassica campestris

Turnips
(contd.)

Purple Top Milan	3-Butenyl isothiocyanate + 4-pentenyl + alyssin + hydrophilic oils + trace goitrin	2-1/2 g. as butenyl/kg.
Purple Top White Globe	3-Butenyl isothiocyanate + 4-pentenyl + hydrophilic oils + goitrin	2 g. as butenyl/kg. 1 g./kg.
Round White	3-Butenyl isothiocyanate (+ trace 4-pentenyl) + alyssin (+ hydrophilic oil) + goitrin	3 g. as butenyl/kg. 1 g./kg.
Seven Top	3-Butenyl isothiocyanate + 4-pentenyl + traces alyssin and sulforaphane (+ hydrophilic oil) + goitrin	3 g. as butenyl/kg. 1 g./kg.
Shogoin	3-Butenyl isothiocyanate + hydrophilic oils	2 g. as butenyl/kg.
Sweet Germaine	3-Butenyl isothiocyanate + 4-pentenyl + (minor) allyl + alyssin + hydrophilic oils + goitrin	3-1/2 g. as butenyl/kg. 2 g./kg.

(cor.td.)

Brassica campestris

Turnips (contd.)

White Egg	3-Butenyl isothiocyanate	5 g. as butenyl/kg.
	+ 4-pentenyl	
	+ minor hydrophilic oils	
	+ goitrin	1 g./kg.

Italian Turnip

Rapa Broccoli (Ferry-Morse)	3-Butenyl isothiocyanate	5 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	+ hydrophilic oils	
	+ trace goitrin	(0.1 g./kg.)

Tendergreen

Tendergreen Spinach-mustard	3-Butenyl isothiocyanate	3 g. as butenyl/kg.
	(+ hydrophilic oil)	
Tendergreen Mustard (Vaughan)	3-Butenyl isothiocyanate	1 g. as butenyl/kg.
	+ 4-pentenyl + allyl	
	+ sulforaphane + alyssin	
	(+ hydrophilic oil)	

Chinese Cabbages

Chihili	3-Butenyl isothiocyanate	1/2 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	+ hydrophilic oils	
	+ goitrin	1/2 g./kg.

(contd.)

Brassica campestris

Chinese Cabbages
(contd.)

Michihli	3-Butenyl isothiocyanate	2-1/2 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	+ hydrophilic oils	
	+ goitrin	1 g./kg.
Mandarin	3-Butenyl isothiocyanate	1-1/2 g. as butenyl/kg.
	+ 4-pentenyl	
	+ alyssin (minor) + trace sulforaphane	
	(+ trace goitrin)	
Paoting	3-Butenyl isothiocyanate	2 g. as butenyl/kg.
	+ trace alyssin + other oils	
Pe-Tsai	3-Butenyl isothiocyanate	1-1/2 g. as butenyl/kg.
	+ 4-pentenyl	
	+ alyssin + trace sulforaphane	
	+ goitrin	1 g./kg.
Wong Bok	3-Butenyl isothiocyanate	2-1/2 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	(+ hydrophilic oil)	
Chinese Cabbage (Sordillo)	3-Butenyl isothiocyanate	3 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	+ sulforaphane + other oils	

(contd.)

Brassica campestris

Chinese Cabbages
(contd.)

Japanese Cabbage (Sordillo)	3-Butenyl isothiocyanate + 4-pentenyl + alyssin + sulforaphane + other oils	1-1/2 g. as butenyl/kg.

Brassica juncea

Foliage, Oriental, and Brown Mustards

Oriental (French Co.)	Allyl isothiocyanate	6 g./kg.
Brown (French Co.)	Allyl isothiocyanate	8 g./kg.
Chinese Smooth Leaf	Allyl isothiocyanate	6-1/2 g./kg.
Chinese Broad Leaf	Allyl isothiocyanate (+ trace hydrophilic oil)	7-1/2 g. as allyl/kg.
Florida Broad Leaf	Allyl isothiocyanate	6-1/2 g./kg.
Giant Southern Curled	Allyl isothiocyanate (+ trace hydrophilic oil)	5 g. as allyl/kg.
Ostrich Plume	Allyl isothiocyanate + trace hydrophilic oil	8 g. as allyl/kg.
Green Wave	Allyl isothiocyanate (+ trace hydrophilic oil)	2-1/2 g. as allyl/kg.
Fordhook Fancy (Burpee)	Allyl isothiocyanate + traces 3-butenyl and three hydrophilic oils	4 g. as allyl/kg.
Fordhook Fancy (Reuter)	Allyl isothiocyanate + 3-butenyl + traces three hydrophilic oils	7 g. as allyl/kg.

Brassica napus

Winter Rape

Dwarf Essex	3-Butenyl isothiocyanate + traces alyssin and sulforaphane (and hydrophilic oil) + goitrin	2 g. as butenyl/kg. 3 g./kg.

Rape-kale

Siberian Kale	3-Butenyl isothiocyanate + 4-pentenyl + alyssin + sulforaphane (+ trace hydrophilic oil) + goitrin	3-1/2 g. as butenyl/kg. 3 g./kg.
Dwarf Siberian Kale	3-Butenyl isothiocyanate + allyl (trace) + 4-pentenyl + hydrophobic oil (possibly nasturtiin or berteroin) + alyssin + sulforaphane + goitrin	2 g. as butenyl/kg. 3 g./kg.
Blue Siberian Kale	3-Butenyl isothiocyanate + allyl + (trace) 4-pentenyl + alyssin + sulforaphane + goitrin	1/2 g. as butenyl/kg. 3 g./kg.

(contd.)

Brassica napus

Rutabagas

American Purple Top	Alyssin	1-1/2 g. as alyssin/kg.
	(+ hydrophilic oil)	
	+ goitrin	5 g./kg.
Laurentian	Alyssin	2 g. as alyssin/kg.
	(+ hydrophilic oil)	
	+ goitrin	7 g./kg.
Purple Top Yellow	3-Butenyl isothiocyanate	1/2 g. as butenyl/kg.
	+ alyssin + trace sulforaphane	
	+ goitrin	4-1/2 g./kg.

Brassica nigra

Black Mustard

Italian (French Co.)	Allyl isothiocyanate (+ trace hydrophilic oil)	8-1/2 g. as allyl/kg.
Turkish (French Co.)	Allyl isothiocyanate	10 g./kg.

	Brassica oleracea	
	Thousand-headed Kale	
Jersey (Ferry-Morse)	Allyl isothiocyanate	5-1/2 g. as allyl/kg.
	+ iberin + sulforaphane	
	+ traces 3-butenyl isothiocyanate and hydrophobic oil	
	(+ hydrophilic oil)	
	+ goitrin	3 g./kg.
	Collards	
Heading Buncombe (Cabbage-collards)	Allyl isothiocyanate	4 g. as allyl/kg.
	+ 3-butenyl	
	+ iberin + sulforaphane	
	+ traces ibervirin and erucin	
	(+ hydrophilic oil)	
	+ goitrin	3 g./kg.
Louisiana Sweet	Allyl isothiocyanate	2 g. as allyl/kg.
	+ 3-butenyl (minor)	
	+ iberin (+ hydrophilic oil)	
	+ goitrin	5 g./kg.
New Georgia (Cabbage-collards)	Allyl isothiocyanate	2-1/2 g. as allyl/kg.
	+ 3-butenyl (minor)	
	+ iberin (+ hydrophilic oil)	
	+ goitrin	7 g./kg.

(contd.)

Brassica oleracea

Collards (contd.)

True Georgia	Allyl isothiocyanate	5 g. as allyl/kg.
	+ 3-butenyl (trace)	
	+ iberin + sulforaphane	
	(+ hydrophilic oil)	
	+ goitrin	4-1/2 g./kg.
Vates	Allyl isothiocyanate	4 g. as allyl/kg.
	+ iberin (+ hydrophilic oil)	
	+ goitrin	6 g./kg.

Cabbages, red

Mammoth Red Rock	Allyl isothiocyanate	4 g. as allyl/kg.
	+ 3-butenyl	
	+ iberin + sulforaphane	
	+ traces ibervirin and erucin	
	(+ hydrophilic oil)	
	+ goitrin	4 g./kg.
Red Acre	Allyl isothiocyanate	4 g. as allyl/kg.
	+ 3-butenyl	
	+ iberin + sulforaphane	
	+ (minor) ibervirin and erucin	
	(+ hydrophilic oil)	
	+ goitrin	4-1/2 g./kg.

(contd.)

Brassica oleracea

Cabbages, red (contd.)

Red Erfurt	Allyl isothiocyanate	5 g. total as allyl/kg.
	+ iberin + sulforaphane	
	(+ hydrophilic oil)	
	+ goitrin	4 g./kg.

Cabbages, white

All Head Early	Allyl isothiocyanate	5 g. total as allyl/kg.
	+ 3-butenyl (trace)	
	+ iberin + sulforaphane	
	+ ibervirin (minor)	
	+ erucin (trace)	
	(+ hydrophilic oil)	
	+ goitrin	1 g./kg.
Bugner	Allyl isothiocyanate	4 g. as allyl/kg.
	+ iberin + sulforaphane	
	+ ibervirin + trace erucin	
	(+ hydrophilic oil)	
	+ goitrin	1/2 g./kg.

(contd.)

Brassica oleracea

Cabbages, white (contd.)

Copenhagen Market	Allyl isothiocyanate	6-1/2 g. total as allyl/kg.
	+ 3-butenyl (trace)	
	+ iberin + (minor)	
	sulforaphane	
	+ ibervirin (minor)	
	+ trace erucin	
	(+ hydrophilic oil)	
	+ goitrin	1 g./kg.
Danish Ballhead Short Stem	Allyl isothiocyanate	2 g. as allyl/kg.
	+ iberin + (minor)	
	sulforaphane and	
	ibervirin + trace erucin	
	(+ trace goitrin	0.2 g./kg.)
Early Flat Dutch	Allyl isothiocyanate	4-1/2 g. total as allyl/kg.
	+ iberin + sulforaphane	
	+ ibervirin (minor)	
	+ trace erucin	
	(+ hydrophilic oil)	
	+ goitrin	1 g./kg.

(contd.)

Brassica oleracea

Cabbages, white (contd.)

European Early Summer	Allyl isothiocyanate	3-1/2 g. total as allyl/kg.
	+ 3-butenyl (minor)	
	+ iberin + sulforaphane	
	+ (minor) ibervirin and	
	erucin (+ hydrophilic oil)	
	+ goitrin	1 g./kg.
Fottler's Improved Brunswick	Allyl isothiocyanate	5 g. total as allyl/kg.
	+ 3-butenyl (minor)	
	+ iberin + sulforaphane	
	+ (minor) ibervirin and	
	erucin (+ hydrophilic oil)	
	+ goitrin	1 g./kg.
Globe	Allyl isothiocyanate	4-1/2 g. as allyl/kg.
	+ iberin + (minor)	
	sulforaphane and ibervirin	
	(+ hydrophilic oil)	
	+ trace goitrin	(0.2 g./kg.)
Glory of Enkhuizen	Allyl isothiocyanate	6 g. total as allyl/kg.
	+ iberin + sulforaphane	
	(+ hydrophilic oil)	
	+ goitrin	2 g./kg.

(contd.)

Brassica oleracea

Cabbages, white (contd.)

Golden Acre	Allyl isothiocyanate	1 g. as allyl/kg.
	+ 3-butenyl (trace)	
	+ iberin + sulforaphane	
	+ (minor) ibervirin and erucin	
	+ goitrin	2 g./kg.
Jersey Wakefield	Allyl isothiocyanate	5 g. total as allyl/kg.
	+ 3-butenyl (minor)	
	+ iberin and sulforaphane	
	+ (minor) ibervirin and erucin	
	(+ trace hydrophilic oil)	
	+ goitrin	2-1/2 g./kg.
Marion Market	Allyl isothiocyanate	6 g. total as allyl/kg.
	+ iberin + (minor) sulforaphane	
	(+ hydrophilic oil)	
	+ goitrin	1-1/2 g./kg.
Premium Flat Dutch	Allyl isothiocyanate	2 g. as allyl/kg.
	+ iberin + sulforaphane	
	+ ibervirin + trace erucin	
	(+ hydrophilic oil)	
	+ trace goitrin	(0.2 g./kg.)

(contd.)

Brassica oleracea

Cabbages, white (contd.)

Wisconsin All Seasons	Allyl isothiocyanate	4 g. as allyl/kg.
	+ iberin	
	+ (minor) ibervirin	
	(+ hydrophilic oil)	

Savoy Cabbages

Chieftain	Allyl isothiocyanate	1-1/2 g. as allyl/kg.
	+ iberin + sulforaphane	
	+ ibervirin + trace erucin	
	+ minor hydrophilic oils	
	(+ trace goitrin)	
Drumhead	Iberin	6 g. as iberin/kg.
	+ sulforaphane	
	+ allyl isothiocyanate	
	+ ibervirin + trace erucin	
	(+ hydrophilic oil)	
	+ goitrin	1/2 g./kg.
European Drumhead	Allyl isothiocyanate	4-1/2 g. total as allyl/kg.
	+ 3-butenyl (trace)	
	+ iberin + sulforaphane	
	+ ibervirin (minor)	
	+ trace erucin	
	+ goitrin	1 g./kg.
(contd.)		

Brassica oleracea

Savoy Cabbages (contd.)

Perfection Drumhead	Allyl isothiocyanate	5 g. total as allyl/kg.
	+ iberin	
	+ ibervirin (minor)	
	+ erucin (trace)	
	(+ trace hydrophilic oil)	
	+ goitrin	1 g./kg.

Kales, curled or variegated

Blue Curled Scotch	Allyl isothiocyanate	5 g. as allyl/kg.
	+ iberin + (minor)	
	sulforaphane	
	+ ibervirin (+ trace erucin)	
	(+ trace goitrin	0.2 g./kg.)
Dwarf Curled Scotch	Allyl isothiocyanate	5-1/2 g. total as allyl/kg.
	+ iberin + sulforaphane	
	+ ibervirin (minor)	
	(+ hydrophilic oil)	
Dwarf Green Curled Scotch	Allyl isothiocyanate	4 g. total as allyl/kg.
	+ iberin + (minor)	
	sulforaphane and	
	ibervirin	
	(+ hydrophilic oil)	
	+ goitrin	1 g./kg.

(contd.)

Brassica oleracea

Kales, curled or variegated (contd.)

Tall Curled Scotch	Allyl isothiocyanate + iberin + sulforaphane + ibervirin (minor) + trace erucin (+ trace goitrin	4 g. total as allyl/kg. 0.1 g./kg.)
Tall Green Curled	Allyl isothiocyanate + 3-butenyl (minor) + iberin + sulforaphane + ibervirin (minor) + trace erucin + goitrin	3 g. as allyl/kg. 1 g./kg.
Christmas Variegated	Allyl isothiocyanate + iberin + (minor) ibervirin (+ trace erucin) (+ hydrophilic oil)	5 g. as allyl/kg.
Flowering Variegated	Allyl isothiocyanate + iberin + (minor) sulforaphane and ibervirin	4 g. as allyl/kg.

(contd.)

	Brassica oleracea	
	Brussels Sprouts	
Catskill	Allyl isothiocyanate	2 g. as allyl/kg.
	+ 3-butenyl (trace)	
	+ iberin + sulforaphane	
	+ ibervirin + erucin	
	+ goitrin	1/2 g./kg.
Long Island	Allyl isothiocyanate	3-1/2 g. total as allyl/kg.
	+ iberin + sulforaphane	
	(+ trace hydrophilic oil)	
	+ goitrin	2-1/2 g./kg.
	Cauliflower	
Autumn Giant	Allyl isothiocyanate	7 g. as allyl/kg.
	+ iberin + trace sulforaphane	
	+ ibervirin (minor)	
	(+ trace erucin)	
	(+ hydrophilic oil)	
Dry Weather	Allyl isothiocyanate	5-1/2 g. as allyl/kg.
	+ iberin + trace sulforaphane	
	+ (minor) ibervirin	
	(+ hydrophilic oil)	
	(+ trace goitrin	0.2 g./kg.)
(contd.)		

Brassica oleracea

Cauliflower
(contd.)

Early Snowball	Allyl isothiocyanate + iberin + (minor) ibervirin (+ hydrophilic oil)	4-1/2 g. total as allyl/kg.
Ideal	Allyl isothiocyanate + iberin + trace sulforaphane + ibervirin	4 g. as allyl/kg.
Master Original	Allyl isothiocyanate + iberin + ibervirin (+ trace hydrophilic oil) (+ trace goitrin)	5-1/2 g. as allyl/kg.
Original Helios	Allyl isothiocyanate + iberin + (minor) ibervirin and hydrophobic oil (+ hydrophilic oil)	5 g. total as allyl/kg.

(contd.)

Brassica oleracea

Kohlrabi

Early Purple Vienna (Burpee)	Iberin + sulforaphane + trace alyssin + allyl isothiocyanate + traces 3-butenyl and iberverin + (minor) erucin	6 g. total as iberin/kg.
	+ goitrin	1 g./kg.
Early Purple Vienna (Sordillo)	Iberin + sulforaphane + allyl isothiocyanate + (minor) ibervirin and erucin (+ hydrophilic oil)	6-1/2 g. total as iberin/kg.
	+ goitrin	1/2 g./kg.
Early White Vienna	Iberin + sulforaphane + (minor) allyl isothiocyanate, ibervirin and erucin	3 g. total as iberin/kg.
	+ goitrin	1/2 g./kg.
White Vienna	Iberin + sulforaphane + (minor) allyl isothiocyanate, ibervirin and erucin + hydrophilic oils	8 g. total as iberin/kg.
	+ goitrin	1 g./kg.

(contd.)

Brassica oleracea

Broccoli

Calabrese	Sulforaphane + erucin + trace alyssin (+ traces allyl isothiocyanate and ibervirin)	6 g. as sulforaphane/kg.
De Cicco	Sulforaphane + erucin + trace 3-butenyl isothiocyanate (or ibervirin) (+ trace hydrophilic oil)	9-1/2 g. as sulforaphane/kg.
Green Sprouting	Sulforaphane + erucin + iberin + trace 3-butenyl isothiocyanate (or ibervirin) + trace goitrin	6 g. as sulforaphane/kg. 1/2 g./kg.

Brassica incert. sed. (Italian vegetables, Sordillo)

Broccoli	3-Butenyl isothiocyanate + 4-pentenyl + sulforaphane + (minor) alyssin and iberin (+ hydrophilic oil)	5 g. total as butenyl/kg.
Broccoletti di Rape	3-Butenyl isothiocyanate + 4-pentenyl + alyssin + minor hydrophobic oil	2 g. as butenyl/kg.
Autumno Broccoli di Rape	3-Butenyl isothiocyanate + 4-pentenyl + alyssin + goitrin	4 g. as butenyl/kg. 1/2 g./kg.
Italian Broccoli Rape	3-Butenyl isothiocyanate + 4-pentenyl + alyssin + minor hydrophobic oil (+ hydrophilic oil) + trace goitrin	3-1/2 g. as butenyl/kg. (0.2 g./kg.)
Rape Italiane	3-Butenyl isothiocyanate + 4-pentenyl + trace alyssin (+ hydrophilic oil) + trace goitrin	2 g. as butenyl/kg. (0.2 g./kg.)

(contd.)

Brassica incert. sed. (Italian vegetables, Sordillo) (contd.)

Cavolo Senza Testa	Allyl isothiocyanate	4-1/2 g. as allyl/kg.
	+ 3-butenyl (minor)	
	+ iberin + sulforaphane	
	+ (minor) ibervirin	
	+ trace erucin (+ hydrophilic oil)	
	+ trace goitrin	(0.2 g./kg.)
Cavolofiore Verde	Iberin + sulforaphane	5 g. total as iberin/kg.
	+ allyl isothiocyanate	
	+ (minor) ibervirin and erucin	
	(+ hydrophilic oil)	
Cavolo Broccolo	Sulforaphane	9 g. as sulforaphane/kg.
	+ erucin	
	+ traces other hydrophobic oils	
Cimmo di Cavolo Noro	Sulforaphane	6-1/2 g. as sulforaphane/kg.
	+ erucin	
	+ traces allyl isothiocyanate and other hydrophobic oils	

Brassica incert. sed.

Brassica carinata A. Braun (Rome)	Allyl isothiocyanate	8-1/2 g. as allyl/kg.
	+ hydrophilic oil	
Brassica japonica Sieb. (Rome)	3-Butenyl isothiocyanate	2 g. total as butenyl/kg.
	+ sulforaphane + alyssin	
	+ goitrin	5 g./kg.
Brassica campestris (Mexico, Fearing #2014)	3-Butenyl isothiocyanate	3 g. as butenyl/kg.
	+ allyl	
	+ minor hydrophobic oil	
Brassica (source unknown, probably bird rape)	3-Butenyl isothiocyanate	2 g. as butenyl/kg.
	+ 4-pentenyl	
Bird Rape	3-Butenyl isothiocyanate	2 g. as butenyl/kg.
	+ 4-pentenyl + alyssin	
	+ sulforaphane	
	(+ hydrophilic oil)	
	+ goitrin	5-1/2 g./kg.
Rape (French Co.)	3-Butenyl isothiocyanate	2 g. as butenyl/kg.
	+ alyssin (+ hydrophilic oil)	
	+ goitrin	5 g./kg.
Brassica (Brazil, Irwin #2145)	Allyl isothiocyanate	10 g. as allyl/kg.
	+ 3-butenyl	
	+ trace hydrophilic oil	

(contd.)

Brassica incert. sed. (contd.)

Californian Black Mustard (Robin)	Allyl isothiocyanate + 3-butenyl + iberin (+ hydrophilic oil)	8-1/2 g. as allyl/kg.
European Mustard (Sordillo)	Allyl isothiocyanate + 3-butenyl + iberin + hydrophilic and hydrophobic oils	6-1/2 g. as allyl/kg.
Perennial Broccoli (Pearce)	Allyl isothiocyanate + iberin + ibervirin (+ trace hydrophilic oil) + trace goitrin	9 g. as allyl/kg. 1/2 g./kg.
Early Purple Head Cauliflower	Sulforaphane + iberin + erucin + trace ibervirin or 3-butenyl isothiocyanate	9 g. as sulforaphane/kg.

Brassicella

Brassicella erucastrum (L.) Schulz [Brassica cheiranthos Vill., from Paris]	Goitrin	5-1/2 g./kg.
Brassicella richeri (Vill.) Schulz [Brassica richeri Vill., from Paris]	Goitrin	10 g./kg.

Diplotaxis

Diplotaxis erucoides (L.) DC., from Paris	Allyl isothiocyanate	3 g./kg.
Diplotaxis erucoides, from Rome	Allyl isothiocyanate + trace 3-butenyl	3-1/2 g. as allyl/kg.
Diplotaxis tenuifolia (L.) DC., from Cologne	Sulforaphane + erucin + trace alyssin	14 g. as sulforaphane/kg.

Eruca sativa

Eruca sativa (Rome)	Erucin + sulforaphane	14 g. as erucin/kg.
Aruchetta (Sordillo)	Erucin + sulforaphane	11 g. as erucin/kg.
Roquette (Reuter)	Erucin + sulforaphane	14-1/2 g. as erucin/kg.

Erucastrum

Erucastrum abyssinicum (Rich.) Schulz, from Rome	Iberin + ibervirin	8-1/2 g. as iberin/kg.
Erucastrum gallicum (Willd.) Schulz, from Paris	Allyl isothiocyanate + trace 3-butenyl + sulforaphene + (both minor) more and less hydrophilic oils as in Raphanus	7 g. total as sulforaphene/kg.
Erucastrum nasturtiifolium (Poiret) Schulz, from Vienna	New mustard oil, giving thiourea after ammonia treatment that on chromatography behaved like nasturtiin or berteroin thioureas in chloroform, but was more hydrophobic than either in 10:1 toluene-butanol	

Hirschfeldia

Hirschfeldia incana (L.) Lagreze-Fossat [Brassica adpressa Boiss., from Paris]	3-Butenyl isothiocyanate + trace allyl	5 g. as butenyl/kg.

Sinapis

Sinapis alba

Yellow Mustard (French Co.)*	p-Hydroxybenzyl isothiocyanate	19 g./kg.

Sinapis arvensis

Charlock

Four samples (Bacigalupi; French Co.; Cologne; Rome)*	p-Hydroxybenzyl isothiocyanate (+ other oils)	12-15 g./kg.
(Paris)	p-Hydroxybenzyl isothiocyanate	20 g./kg.
	+ other oils	2 g. as if nasturtiin/kg.

*Assays by Barbara W. Harrison.

Raphanus

Raphanus maritimus

Raphanus maritimus Smith, from Paris	Sulforaphene	7 g. as if sulforaphene/kg.
	+ more hydrophilic oil	
	+ trace more hydrophobic component (as in R. sativus)	
	+ allyl isothiocyanate (minor)	
	(+) trace thiooxazolidone	1/2 g. as if goitrin/kg.

(contd.)

Raphanus

Raphanus sativus

Radishes

Twelve varieties (Cherry Belle; Chienese Rose Winter; Crimson Giant; French Breakfast; Long Black Spanish; Long Scarlet Short Top; Pith Proof; Scarlet Globe; Scarlet Turnip White Tip; Sparkler White Tip; White Box; White Icicle)*	Sulforaphene	11-17 g./kg.
Long Dark	Sulforaphene	8 g./kg.
Round Dark	Sulforaphene	6 g./kg.
Burpee White	Sulforaphene	8-1/2 g./kg.
Giant Butter	Sulforaphene	8-1/2 g./kg.
White Strassburg Summer	Sulforaphene	11-1/2 g./kg.

The last three radishes contained traces of a thiooxazolidone (1/2 g. as if goitrin/kg.), which apparently behaved like ethylthiourea on chromatography in chloroform but was more hydrophilic in 3:1 butanol-toluene, and a minor oil more hydrophilic than sulforaphene, seemingly identical with the major component from R. maritimus.

Raphanus sativus	Sulforaphene	11-1/2 g. as sulforaphene/kg.
"Raphanus sinensis Mill.," from Rome (sic; Raphanus chinensis Mill.)	+ more hydrophilic oil	
	+ goitrin	1 g./kg.

*Assays by Barbara W. Harrison.

	Crambe	
Crambe cordifolia Steven, from Rome and Vienna	3-Butenyl isothiocyanate	4 g./kg.
	Rapistrum	
Rapistrum rugosum (L.) Allioni, from Paris	Cheirolin	22 g./kg.
	+ trace thiooxazolidone	1/2 g. as if goitrin/kg.
var. (or subsp.) *orientale* (L.) Arcangeli [Crantz], from Rome	Cheirolin	8 g. as cheirolin/kg.
	+ traces allyl isothiocyanate and other oils	

Discussion

Since the results, like playing cards, speak for themselves, comment will be brief. Brassica nigra and B. juncea yield dominantly allyl isothiocyanate as already known. The content in B. juncea seed grown for mustard greens is not much less than in the sorts used as condiment. The Fordhook Fancy strain shows 3-butenyl isothiocyanate. Under B. campestris, turnip seed has 3-butenyl isothiocyanate and frequently goitrin as major constituents, accompanied by 4-pentenyl isothiocyanate. Among the sulfoxides, iberin unexpectedly preponderates over sulforaphane. Allyl isothiocyanate occasionally is a minor component. The Oriental representatives, Chinese cabbages and tendergreen mustard, possess decidedly less goitrin on the average but are instructively similar to the turnips. B. napus generally resembles B. campestris, but in rutabaga seed the goitrin content is high and the mustard oils, 3-butenyl isothiocyanate in particular, notably reduced. The chemical data strongly confirm Musil's judgment[2] on the basis of seed appearance that Siberian kales belong in B. napus, not B. oleracea.

Brassica oleracea, a varied species morphologically, is chemically the most complex. A generalized composition would include allyl isothiocyanate, goitrin, the sulfoxides iberin and sulforaphane, and lesser amounts of 3-butenyl isothiocyanate and the sulfides ibervirin and erucin. The thousand-headed kale and collards, supposed to be among the most primitive types, and the red cabbages are set off by high goitrin content (0.3-0.7%). Whether the amounts of goitrin in seed and leaves are parallel is unknown. The group with much goitrin also shows 3-butenyl isothiocyanate in most

examples and is low in sulfides. The white and Savoy cabbages, curled and variegated kales, and Brussels sprouts have little goitrin (0-0.25%, average less than 0.1%) and consistently yield ibervirin with a little erucin. Cauliflower, overlapping a few cabbages and kales, has a characteristic pattern of great preponderance by the compounds with a three-carbon chain, allyl isothiocyanate, iberin and ibervirin. In kohlrabi, which resembles some cabbages, allyl isothiocyanate is less important than usual in the preceding varieties, and the sulfoxides iberin and sulforaphane stand out. Finally, in broccoli allyl isothiocyanate has substantially disappeared[10] and sulforaphane, accompanied by erucin and perhaps iberin, is the major component.

The Brassica of uncertain position, that is, not belonging to a well recognized American variety, offer a test whether chemical analysis can contribute to the commercially interesting problem of identification from seed and seedling characters.[2,23] The ambiguity of some names is illustrated by the definition[24] of the Italian word broccolo (cf. brocco, shoot or sprout): "broccoli; flower stalk of a turnip; (N. Ital.) cauliflower." Manifestly, the first five Italian vegetables listed, under such titles as "broccoli" or "broccoli rape" as well as rape Italiane, belong to the turnip group like the Italian turnip broccoli previously listed, as B. campestris cultivated for greens. On the other hand, the "cavolo broccolo" and "cimmo di cavolo noro" are true broccoli. The "cavolo senza testa" (headless cabbage) would fit a kale, and the "cavolofiore verde" is a cauliflower-broccoli intermediate, much like kohlrabi. Of American specialties, the "early purple head cauliflower" is clearly a broccoli and the "perennial broccoli" conversely a cauliflower.

The dominance of allyl isothiocyanate in Brassica carinata (Abyssinian mustard, n = 17) befits the plant's status as an amphiploid hybrid of B. nigra and B. oleracea. B. japonica, if properly attributed, is not a variety of B. nigra as Schulz[4] had it but a form of B. campestris (or possibly napus), in accord with the presumable Eastern origin. The Brazilian Brassica and the Californian and European mustards suggest a kind of B. juncea.

If other genera are now considered, the occurrence of goitrin alone in Brassicella [Rhynchosinapis], as well as in Brassica barrelieri, is worth notice. Diplotaxis tenuifolia, yielding much sulforaphane accompanied by erucin, has a composition like that of broccoli. The same two compounds are found in Eruca sativa, but in reverse proportion. Erucastrum, so far as examined, was peculiarly heterogenous. E. abyssinicum was a counterpart of D. tenuifolia with the carbon chains shortened by one unit. E. nasturtiifolium furnished a new mustard oil with thiourea R_{Ph} 1.1 in chloroform, 1.47 in 1:10 butanol-toluene. E. gallicum was most remarkable, for the simultaneous presence in quantity of allyl isothiocyanate and sulforaphene constituted a natural link between the important genera Brassica and Raphanus, a chemical counterpart of an artificial hybrid Raphanobrassica.[25] The radishes themselves were uniform in composition, qualitatively and quantitatively, but one of the minor compounds (R_{Ph} of thiourea 0.27 in 3:1 butanol-toluene) became the principal isothiocyanate in Raphanus maritimus. Finally, attention may be called to the considerable amount of 4-hydroxybenzyl isothiocyanate in charlock, nearly as much as in yellow mustard.

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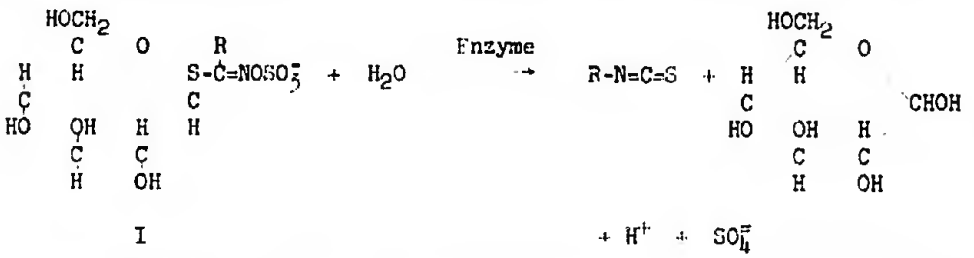
Part II

Mustard and Vitamin C

Introduction

The liberation of natural mustard oils was one of the oldest known enzymatic reactions. Between 1830 and 1840, the character of the process was recognized[1,2] and two substrates, the mustard oil glucosides sinigrin or potassium myronate[2] (I, R = $H_2C{=}CHCH_2$) and sinalbin[3,4] (I, R = p-$HOC_6H_4CH_2$, sinapine salt), were isolated in crystalline form. Sound



inquiry, however, was retarded until the structure of the glucosides was correctly assembled[5] in 1956. Among descriptions of the enzymatic cleavage, no hint of a cofactor appeared until 1959, when Nagashima and Uchiyama[6] announced that the reaction was remarkably promoted by ascorbic acid. They discovered that the rate of hydrolysis of sinigrin by an enzyme preparation from yellow mustard increased by 260% on addition of 0.001 M L-ascorbic acid, whereas an equal concentration of the stereoisomeric D-araboascorbic acid produced a rise of only 50%. With other, crude plant extracts, they found that L-ascorbic acid could accelerate attack on sinigrin by factors as large as 100.

The independent study presented here of the relation between Vitamin C and enzymes (glucosinolases) that decompose mustard oil glucosides (glucosinolates[7]) began in 1958 with observations like those cited. In experiments by Miss Barbara Harrison, the enzymatic activity of yellow

mustard flour (dried, ground and threshed seed of Sinapis alba) was assayed by addition of the solid to buffered sinalbin. When the flour was first kept in concentrated aqueous suspension at room temperature during an hour or more, following accepted procedures for extraction of the crude enzyme[2,8] termed myrosin, only about a third of the activity survived in the mixture and but half of that remainder was soluble. The presence of fluoride, azide or arsenious oxide during maceration was found to furnish more active solutions. Since the first two preservatives could inhibit metal-containing oxidases and azide retarded darkening of the extracts (also, trivalent arsenic was a reducing agent), ascorbic acid was tried. It was shortly discovered that addition of 0.003 M L-ascorbate to mustard flour with sinalbin would accelerate cleavage of the glucoside by a factor of 80. Much higher concentrations of D-araboascorbate were required for a similar effect and D-glucoascorbate was inert. No enzymatic process involving Vitamin C was known of such structural specificity, parallelling nominal antiscorbutic activity.

The response to optimal L-ascorbate in decomposition of mustard oil glucosides by the usual aqueous mustard extracts or myrosin preparations was a rate factor of merely 4 or so.[6,9] Of the ascorbate-activated glucosinolase in the flour before maceration, only 1% was apparent in the liquid. Perhaps half of the wanted enzyme was destroyed and the rest was largely insoluble. Ordinary myrosin was thus unsuited to investigation of the ascorbate effect. We conducted extensive preliminary work on factors controlling the hydrolysis of glucosinolates by whole mustard. A simple procedure was eventually developed for extraction of the ascorbate-activated

glucosinolase, which gave in 40% yield a stable enzyme solution, protein in character and purified over the flour approximately fifteen fold. The enzyme retained the behavior of the native system and the maximal ascorbate effect on glucosinolate cleavage was a rate factor of 300. Experiments at further purification only doubled the specific activity to a level of 300 micromoles of sinigrin hydrolyzed/mg. protein/min. (300 units/mg. protein[10]) at 25° in 0.0015 M L-ascorbate, four hundred times faster than without the vitamin. However, the materials sufficed for reliable detection of less than 10^{-6} M L-ascorbate in presence of 10,000 times as much of its optical antipode and for inferences about the course of reaction of mustard oil glucosides and the part of Vitamin C.

Methods

The hydrolysis of (I) can be followed by determination of substrate or any product. Of course, if intermediates exist, all methods need not give the same rate. Simple techniques were used with glucoside concentrations of 0.006-0.01 M at the start so that the reaction was approximately zero-order.

1) Evolution of mustard oil: Sinalbin in neutral or acidic buffer was shaken with the flour and diethyl ether. The p-hydroxybenzyl isothiocyanate liberated into the ether phase was determined by ultraviolet absorption (in ethanol λmax. 227 mμ, ϵ ca. 11000) and conversion to thiocyanate ion[4,11] with aqueous alkali.

2) Disappearance of glucoside: A buffered solution of sinigrin was stirred with mustard flour or enzyme. Samples were heated swiftly to 95° and evaporated. Absorbancies of the residues were measured in water at 227

mμ, the maximum also of sinigrin (ε 7200).

With enzyme solutions, the hydrolysis of sinigrin could be performed in a cell of 0.5-mm. light path while the absorbance at 228 mμ was recorded by a Cary Model 14 Spectrophotometer.[9] The reference beam was attenuated by screens to measure absorbances above 2. The allyl isothiocyanate produced remained dissolved to 0.01 M with low extinction (ε 600). At high velocities, an unstable, strongly absorbing intermediate appeared which was most evident at longer wave lengths[9] and will be discussed later.

3) Evolution of acid: A glucoside, routinely sinigrin, was cleaved by enzyme in unbuffered solution (0.001 M sodium chloride) with the pH held constant through addition of standard (0.1 M) sodium hydroxide. The process was controlled by a Radiometer Titrator, Titrigraph and syringe burette and the consumption of alkali was recorded against time. Open vessels could be used at pH up to 10.

Nearly all of the work utilized one sample (No. 606) of yellow mustard flour, generously supplied by the R. T. French Company. Similar effects of ascorbate were observed with other specimens of flour and seed of Sinapis alba. Brown mustard (Brassica juncea) flour also contained a conspicuous, ascorbate-activated glucosinolase.

Studies with Whole Mustard

Specific rates of hydrolysis of glucosinolates by yellow mustard flour alone in 0.1 M phosphate (potassium-sodium), pH 6.7, at 30° were 0.06 units/mg. flour for sinalbin and 0.08 for sinigrin. With added 0.0015-0.003 M L-ascorbate (1/4 - 1/2 mg./ml.), the rates were 4.5 (sinalbin) and 7

(sinigrin). Much more ascorbate was inhibitory and at 0.02 M the velocity was roughly half maximal. The rate also fell with ascorbate below 0.001 M and for either glucoside was approximately halved at $2 \cdot 10^{-4}$ M cofactor. (L-Ascorbic acid synthesized from L-xylose had the same coenzymatic property as the commercial vitamin.) The relation of speed to ascorbate concentration below 0.002 M tolerably followed the Michaelis-Menten equation with constants of $2\text{-}3 \cdot 10^{-4}$ M. The concentration giving a total rate double that without vitamin was roughly $3 \cdot 10^{-6}$ M (1/2 µg./ml.). Dilute solutions were protected against aerial oxidation if necessary with D-glucoascorbic acid. Only the reduced state of L-ascorbic acid was a coenzyme.

Tests of numerous enediols and derivatives of Vitamin C showed that the capacity to accelerate enzymatic cleavage of glucosinolates was sharply dependent on structure. L-Ascorbic acid was considerably the most effective compound. For a given active analogue and substrate, a roughly constant ratio often existed between concentrations of L-ascorbate and other cofactor producing the same result. Thus in hydrolysis of sinalbin (35°), $6 \cdot 10^{-4}$ M D-araboascorbate and $3 \cdot 10^{-5}$ M L-ascorbate (ratio 20/1) gave the same velocity and tenfold larger concentrations each furnished a sixfold greater rate. For cleavage of sinigrin (33°), $1.5 \cdot 10^{-3}$ M D-araboascorbate was required to match $3 \cdot 10^{-5}$ M L-ascorbate and 0.03 M D-araboascorbate equalled $4 \cdot 10^{-4}$ M L-ascorbate (ratios 50-75/1). The structural specificity of the enzyme in whole flour appeared the same as in the extract to be described.

Miscellaneous reducing agents and known enzymatic cofactors, activators or inhibitors were examined for effect on the hydrolysis of glucosinolates by mustard flour. At reasonable concentrations the results, save for

possible inhibition by mercurials, were uniformly negative. The following compounds did not appreciably facilitate the cleavage without Vitamin C: hydroquinone, 3,4-dihydroxyphenylalanine, hydroxylamine, hydrogen sulfide, bisulfite, thiosulfate, dithionite, thiourea, cysteine alone or with ethylenedinitrilotetraacetate, penicillamine, mercaptoacetate, and 2,3-dimercaptopropanol. The following substances did not significantly affect the rates of hydrolysis in absence or in presence of L-ascorbate (usually $1.5 \cdot 10^{-4}$ M, sometimes more): glutathione; arsenious oxide; ethylenedinitrilotetraacetate, 8-hydroxyquinoline-5-sulfonate, diethyldithiocarbamate; fluoride, iodide, azide, hydrogen cyanide; inositol, biotin; thiamine pyrophosphate, pyridoxal phosphate; nicotinamide-adenine dinucleotide and its phosphate, oxidized or reduced; flavin mononucleotide, flavin-adenine dinucleotide; adenosine, cytidine, guanosine, inosine and uridine triphosphates; uridinediphosphoglucose; coenzyme A or lipoic acid with glutathione; folic acid; 3-indoleacetic acid, 3-indoleacetonitrile; liver and yeast concentrates (Sigma Chemical Co.); boiled mustard; and a cofactor for sulfate transfer, 3',5'-diphosphoadenosine.

Yellow mustard flour or seed had a copper content of 4-6 µg./g. Since the several complexing agents listed, as well as neutral citrate or pyrophosphate, did not inhibit glucosinolate hydrolysis promoted by Vitamin C, metals other than alkalis were probably not involved.

Although the glucosinolases of whole mustard were routinely studied in 0.1 M phosphate, pH 6.7, neither phosphate nor neutrality was essential to activation. Variation of hydrogen ion concentration over a wide range had no more influence than other salt effects. The rate with sinigrin and

no ascorbate was nearly unaltered in dilute carbonate buffer, pH 9.9, or acetate, pH 4.2, and the speed with 0.003 M vitamin changed from maximal by less than a factor of two. The Michaelis constant of ascorbate also varied little between pH 4 and 10. The promoted reaction formed hydrogen sulfide at pH 4 or less and was trivial at 3. Citrate buffers roughly doubled the speed of cleavage without vitamin but below pH 5 markedly inhibited hydrolysis caused by ascorbate.

Studies with Extracted Enzyme

Preparation.--The ascorbate-activated glucosinolase was but sparingly soluble in neutral salt solutions, even as dilute as 0.01 M. The extraction comprised three parts: treatment of the mustard with a high concentration of a thiol and alkaline buffer to neutralize the mixture; washes with a dilute salt, exhaustive if desired; removal of the enzyme with water. Yellow mustard flour was first defatted with petroleum ether. The insoluble residue (57%) possessed some 75% of the original enzymatic activity.

One gram of defatted flour was stirred five minutes at 0° with 10 ml. of a solution containing 5 ml. of 2-mercaptoethanol, 10 g. of sodium bicarbonate and 5 g. of anhydrous sodium carbonate per liter. The suspension (pH 7-8) was diluted with 10 ml. of ice water and centrifuged (five minutes, 20000 g). The solid was washed once at 0° and three times at 25° with 20-ml. portions of a solution of 0.5 g. of sodium bicarbonate and 0.5 ml. of mercaptoethanol per liter. The final residue was extracted with 20 ml. of 0.01% mercaptoethanol at 25° and centrifuged ten minutes at 20000 g. The supernatant was left overnight at room temperature, clarified two hours at 30000 g and stored at 5°.

The extract, of practical value, contained roughly 1.5 mg. of protein/ml. from the biuret reaction (standardized with bovine serum albumin) or 2 mg./ml. of matter reducing chromate[12] (same standard). The specific activity for hydrolysis of sinigrin at 25° was approximately 150 units/mg. protein in 0.003 M ascorbate, 0.5 without Vitamin C. Dialysis at pH 8 (0.005% triethanolamine--0.01% mercaptoethanol) and recentrifugation (2 hours, 30000 g) gave a solution of 1 mg. of protein/ml., specific

activity 200. Similar treatment but at pH 7 (10^{-4} M phosphate) or 6.5 afforded supernatants respectively with 0.6 and 0.3 mg. of protein/ml., activities 270 and 300. After further centrifugation of the solution of pH 8 for two hours at 140000 g, 40% of the enzyme remained in the liquid with specific activity essentially unaltered. The material was less soluble or dispersed at lower pH and impurities were more readily sedimented.

The enzyme could also be purified, as well as concentrated, in the following manner. When the extract was made up at 0° to 0.01 M in phosphate, pH 7, some 80% of the activity was precipitated. The precipitate could be redispersed in a small volume (0.005% mercaptoethanol--10^{-4} M phosphate) and centrifuged briefly to furnish a solution with as much as 8 mg. of protein/ml. More centrifugation, for several hours at 30000 g, threw down all but roughly 1 mg./ml. The enzyme remaining dissolved could hydrolyze 300-350 micromoles of sinigrin/min./mg. protein at 25° in 0.003 M ascorbate, 0.8 without cofactor. When a purified preparation was kept six months at 5°, the soluble material decreased to 0.7 mg./ml. but the specific activity was unchanged.

In purified enzyme solutions, the protein content and total organic matter[12] agreed within 10%. The ultraviolet spectrum had a maximum at 277-278 mμ, average absorbancy 1.6 (1 mg./ml., 1 cm.), and minimum at 253-255 mμ, absorbancy 1.2 (1.3 and 1.6 at 260 and 280 mμ). The enzyme passed directly through Sephadex G-75 gel, which retards materials of molecular weights up to 40000.

performance of the enzyme is illustrated by Figures 1-3.

Kinetic Conditions.--The speed of sinigrin hydrolysis (at 0.006-0.01 M) increased with enzyme concentration though not so much as linearly. Deviation from proportionality was hopefully disregarded as far as possible. The specific rates, measured by titration in $5 \cdot 10^{-6}$ to 0.002 M ascorbate, appeared to decrease by 10-50% when the enzyme was raised from 1 to 20 µg./ml.

Salt effects were small. Velocities with 0.002 M ascorbate determined spectrophotometrically in 0.1 M phosphate, pH 6.7, or titrimetrically in unbuffered medium, pH 7, differed by no more than 25%. Sulfate did not inhibit up to 0.05 M. The enzyme needed no alkali cation, for the cleavage of sinalbin in water was not appreciably slowed when tetraethylammonium hydroxide served to neutralize the vitamin and monitor progress.

The rate of enzymatic hydrolysis of sinigrin in 0.001 M sodium chloride and 0.0025 M ascorbate varied no more than 10% from pH 6 to 10. The speed fell to roughly 60% of optimal (pH 7-8) at pH 5, 20-30% at 4 and 10-15% (initially; 0.009 M ascorbic acid) at 3.5. With $1.5 \cdot 10^{-6}$ M ascorbate, the rate changed less than 20% between pH 5 and 9.

The velocities were compared of enzymatic attack on sinigrin, progoitrin (I, R = $H_2C{=}CHCHOHCH_2$), glucotropaeolin (I, R = $C_6H_5CH_2$), sinalbin, glucocapparin (I, R = CH_3) and phenylglucosinolate (I, R = C_6H_5), all at 0.006-0.008 M in 0.0025 M ascorbate. The initial rates diminished in the order given, but by no more than a third for all but the last glucoside. Hydrolysis of the phenylglucosinolate was slower than that of sinigrin by a factor of ten but led as normally to phenyl isothiocyanate.[7]

Figs. 1-3.--Titrigraph records of hydrolysis of sinigrin (0.006 M) in 6 ml. of 0.001 M sodium chloride at 27°, pH 7. Each reaction was started by addition of 0.1 mg. of glucosinolase in 0.1 ml. of water.

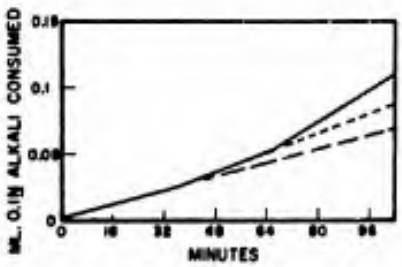


Fig. 1.--No cofactor was present initially. At 35 minutes, 7.7 mg. of sodium D-ascorbate (to make $5.7 \cdot 10^{-3}$ M) was added in 0.8 ml. of water; at 67 minutes, 0.66 μg. of L-ascorbic acid (to make $5.5 \cdot 10^{-7}$ M) in 2.2 μl. The successive slopes were 0.7, 0.99 and 1.5 μl./min.

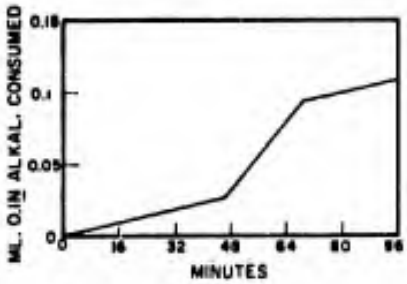


Fig. 2.--No cofactor present initially. At 47 minutes, 2.1 μg. of L-ascorbic acid (to make $2 \cdot 10^{-6}$ M) in 2.1 μl. of water was added; at 69 minutes, 10 μl. of ascorbic oxidase solution (ca. 10 units). The slopes were 0.6, 3 and 0.6 μl./min.

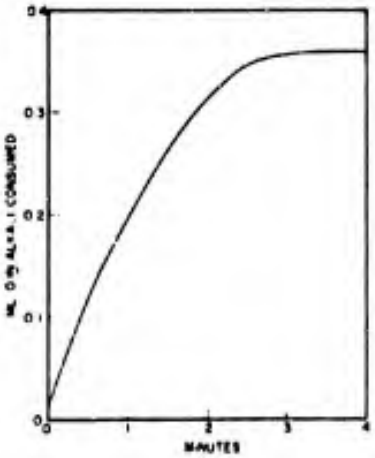


Fig. 3.--Present at start, 3 mg. of sodium L-ascorbate ($2.5 \cdot 10^{-3}$ M) as well as 15 mg. (36 μequiv.) of sinigrin. The consumption of base was 0.20 ml. after 1 minute, 0.315 ml. after 2 minutes, 0.355 ml. after 3 and 0.36 ml. (36 μequiv.) after 3.3-4 minutes.

Michaelis Constants of Sinigrin.--The concentrations of glucoside at which the rates of enzymatic cleavage were reduced to half of the limiting values were determined spectrophotometrically in 0.1 M phosphate, pH 6.7, at 23.5-25°. The results depended on the concentration of L-ascorbate. In 0.001-0.003 M coenzyme, producing maximal velocity, the constant for sinigrin was approximately 0.0015 M, without buffer as well. For ascorbate near its own Michaelis constant, the sinigrin value fell to $7 \cdot 10^{-4}$ M. With little or no vitamin, the results appeared to vary in the same direction as enzyme concentration. Roughly, the Michaelis constants of sinigrin for the promoted reaction, followed in a 2-mm. cell, on extrapolation with ascorbate 10^{-5} M and below converged to the range $5 \cdot 10 \cdot 10^{-5}$ M. The glucoside coefficient for hydrolysis without cofactor, observed with the same amounts of enzyme in a 1-cm. cell, was least of all, on the order 10^{-5} M.

Temperature Effects.--Rates of enzymatic hydrolysis of sinigrin (0.006-0.01 M, pH 7) in absence or presence of L-ascorbate were measured by titration at 0.3° and at 5° intervals from 10° to 65-70°, where denaturation became unduly rapid. For each temperature, the coenzyme-dependent reaction was characterized by the greatest observable velocity and an apparent Michaelis constant, the vitamin concentration furnishing half the maximum rate. Both numbers at 25° were 20-30% below coefficients of the Michaelis equation for cofactor, but trends were probably significant.

Extreme rates and Michaelis constant increased monotonically with temperature. The Arrhenius plot of the enzymatic reaction without ascorbate was closely linear from 15° to 40-45°, showing an activation energy of 8-1/2 kcal./mole. The plot for the cleavage maximally assisted by

vitamin had one linear segment from 0° to 20°, activation energy 17 kcal./mole, and another from 20° to 45°, energy 10-1/2 kcal./mole. Temperature effects on both reactions were small above 50-55°. The apparent Michaelis constant for ascorbate on similar representation gave a curved graph but was fitted by a straight line within 20% of experimental values from 10° to 60°. The indicated heat was 10-15 kcal./mole.

Fastest hydrolysis of sinigrin practically could be achieved at 50° in 0.005 M L-ascorbate. The specific activity, triple optimal at 25°, slightly exceeded 1000 units/mg. protein.

Since thermal variations of maximal velocity and Michaelis constant of ascorbate were similar, rates at low concentrations were relatively independent of temperature. From 45° to 15°, the decrease of speed was 6% in $1.5 \cdot 10^{-5}$ M ascorbate but 75-85% without vitamin or at optimal activation. The detection of trace ascorbate would be easiest near 10°. When a solution of sinigrin and enzyme at 8°, already $2 \cdot 10^{-4}$ M in L-gluconascorbate, was treated with 0.02 µg. of L-ascorbate/ml. (10^{-7} M), the velocity of cleavage increased by 40%.

Vitamin C and Analogues.--The specific rate of hydrolysis of 0.01 M sinigrin determined titrimetrically at pH 7 and 25° with a standard enzyme is shown in Table IV as a function of L-ascorbate concentration. The values are compared with those from a Michaelis-Menten expression plus the rate without cofactor. For a Michaelis constant of $2.5 \cdot 10^{-4}$ M (45 µg./ml.) the experimental and calculated results agree to 15% over a 400-fold range of velocity and a 2500-fold variation of coenzyme, from $6 \cdot 10^{-7}$ to 0.0015 M.

The obedience to the Michaelis law must be qualified. The greatest speed in Table IV, five sixths of the limiting value, was the highest observed. At more than 0.0015 M L-ascorbate, the rate held stationary to perhaps 0.003 M and thereafter diminished, falling at 0.03 M to one third of maximal. Optimal concentrations increased with temperature like the Michaelis constant. Inhibition by excess coenzyme parallelled catalytic efficiency and appeared for less active compounds at higher concentrations or not at all. The velocity with 0.0015 M L-ascorbate was unaffected by 0.02 M D-glucoascorbate and even 0.045 M D-glucoascorbate caused a depression of less than 10%.

Table IV

Hydrolysis with L-Ascorbate

Concentration, ug./ml. x	Observed specific rate, units/mg. protein	Calculated rate $= 0.7 + \frac{360}{1 + \frac{45}{x}}$
0	0.7	0.7
0.1	1.5	1.5
0.2	2.5	2.3
0.3	3.3	3.1
0.5	4.8	4.7
0.7	6.4	6.2
1	8.5	8.5
2	16	16
3	20	23
5	38	37
7	43	49
10	63	66
15	80	91
20	115	111
30	140	145
40	167	170
60	200	206
80	230	231
100	245	249
120	265	263
150	285	278
250	295	306

Another complication was the effect of enzyme concentration on specific rate. In order to keep the experimental velocity within sensible bounds, the enzyme was varied by twenty fold, affecting the data from 1 to 20 µg. of ascorbate/ml. The quantitative concordance of Table IV might be an artifice, although easy to find.

When the relations between specific rate and concentration of analogues of L-ascorbate were determined with the identical method and enzyme, eight substances were found which followed equations with greater Michaelis constants than L-ascorbate but the same upper limiting velocity. The compounds, in classical nomenclature, were L-rhamnoascorbic acid (Table V), 6-desoxy-L-ascorbic acid, D-araboascorbic acid (Table VI), DL-4-ethyl-2-hydroxy-tetronic acid (5,6-bisdesoxy-DL-ascorbic acid) (Table VI), L-gluconascorbic acid, D-erythroascorbic acid, DL-2-hydroxy-4-isopropyltetronic acid and 2-O-methyl-L-ascorbic acid (Table VII). No substance furnished a higher limiting speed or lower Michaelis constant than L-ascorbate, but analogues with smaller maximal rates appeared possibly to be numerous. The clearest example was 5,6-isopropylidene-L-ascorbic acid, having a Michaelis constant of only $5.5 \cdot 10^{-4}$ M but an upper velocity 45% of that for L-ascorbate.

For a compound with the same utmost speed as L-ascorbate, relative coenzymatic activity is defined as the quotient of the Michaelis constant of ascorbate by that of the analogue. The activity is the fixed ratio of concentrations (arbitrarily on a weight, rather than weight-molecular, basis) of L-ascorbate or the other factor producing the same coenzymatic effect. If the limiting velocity of the analogue is not the same as the standard or is impracticable to determine, the measure chosen as abbreviation is the estimated relative slope at low values of the graph of rate of

Table V

Hydrolysis with L-Rhamnoascorbate

Concentration, µg./ml. = x	Observed specific rate, units/mg. protein	Calculated rate $= 0.7 + \frac{360}{1 + \frac{270}{x}}$
0.5	1.6	1.4
1	2.4	2.0
2.5	4.8	4.0
5	6.2	7.2
10	12	14
12.5	15	17
25	31	31
50	51	57
125	106	115
250	183	174
500	233	234
1000	270	284

Table VI

Hydrolysis with D-Araboascorbate or (starred values)

DL-4-Ethyl-2-hydroxytetronate

Concentration, µg./ml. = x	Observed specific rate, units/mg. protein		Calculated rate $= 0.7 + \frac{360}{1 + \frac{2700}{x}}$
25	3.6	*4.5	4.0
50	6.9	*7.0	7.2
100	13		14
130		*17	17
250	31	*29	31
500	55	*51	57
1000	100	*97	98
1750		*143	142
2600	180	*170	177
4900	228		233

Table VII

Hydrolysis with 2-O-Methyl-L-ascorbate

Concentration, μg./ml. = x	Observed specific rate, units/mg. protein	Calculated rate $= 0.7 + \frac{360}{1 + \frac{7800}{x}}$
20	2.0	1.6
50	3.2	3.0
100	5.4	5.3
250	11.5	12
500	23.5	22
1000	39	42
2500	83	88
5000	142	141
9600	200	199

enzymatic hydrolysis (always supposed zero-order) as a function of cofactor concentration. The activity so observed would on assumption of a Michaelis equation be the quotient of coenzymatic constants divided by that of limiting speeds.

In Table VIII are gathered the approximate coenzymatic activities of analogues of Vitamin C, determined titrimetrically with purified glucosinolase and sinigrin (0.01 M) at 25° and, unless stated, pH 7. The Michaelis coefficient ratios are given parenthetically if assignable, though crudely for some. With the least active compounds, salt effects on the ascorbate-independent enzyme may confuse any catalytic power. Figure 1 indicates the meaning of the smallest activities. We have also listed antiscorbutic efficacies, taken from the older literature as the quotient of doses of L-ascorbic acid or analogue needed to maintain guinea pigs. Several compounds with apparent antiscorbutic properties, including the 5,6-isopropylidene, 5,6-diacetyl, 1-O-methyl and 3-O-methyl derivatives of Vitamin C, dehydroascorbic acid and methyl 2-keto-L-gulonate, are possibly or certainly converted in the animal to L-ascorbate.

The relative coenzymatic activities probably depend on temperature, medium and state of the enzyme, but not critically.

Table VIII

Coenzymatic and Antiscorbutic Activities (L-Ascorbate = 1)

Substance	Coenzymatic Activity	Antiscorbutic Potency
Triose reductone	1/1500	<1/20 [13]
Dihydroxyfumaric acid	$<10^{-4}$	< 1/20 [14]
Reductic acid	1/3000	None detected[15]
2-Hydroxytetronic acid	1/400 (1/80)	< 1/20 [16]
DL-2-Hydroxy-4-methyltetronic acid	1/170 (1/70)	
DL-4-Ethyl-2-hydroxytetronic acid	1/60 (1/60)	
DL-2-Hydroxy-4-n-propyltetronic acid	1/60 (1/30)	
DL-2-Hydroxy-4-isopropyltetronic acid	1/90 (1/90)	
DL-4-n-Butyl-2-hydroxytetronic acid	1/150 (1/40)	
DL-2-Hydroxy-4-m-nitrophenyltetronic acid	1/70 (1/20)	
D-Erythroascorbic acid	1/85 (1/85)	1/3 - 1/4 [17]
L-Erythroascorbic acid	$<10^{-4}$	< 1/40 (?) [17]
6-Desoxy-L-ascorbic acid	1/11 (1/11)	1/3 [18]
D-Ascorbic acid	$\leq 10^{-4}$	<1/40 [14,19,20]
D-Araboascorbic acid	1/60 (1/60)	1/20 [16,19-21]
L-Araboascorbic acid	1/5000	<1/40 [19,20]
L-Fucoascorbic acid	1/40 [a]	1/50 - 1/100 [20]
L-Rhamnoascorbic acid	1/6 (1/6)	1/5 - 1/10 [20,22]
D-Alloascorbic acid	1/2000	

(contd.)

Table VIII
(contd.)

D-Galactoascorbic acid	$<10^{-4}$	$<1/40$ [19,20,23]
L-Galactoascorbic acid	1/1500	1/60 [24]
D-Glucoascorbic acid	$<10^{-4}$	$<1/40$ [14,19-21,23]
L-Glucoascorbic acid	1/70 (1/70)	1/40 [20,23]
D-Guloascorbic acid	1/1400	
L-Guloascorbic acid	1/2500	$<1/40$ [19,20]
L-Xyluronoascorbic acid[25]	$<10^{-4}$ (pH 5-7)	
5,6-Isopropylidene-L-ascorbic acid	1/6 (1/3)	1/2 - 1/3 orally; $<1/3$ subcutaneously [26]
5,6-Diacetyl-L-ascorbic acid	1/80 (1/60)	1 [27]
1-O-Methyl-L-ascorbic acid	1/5000 [b]	$>1/4$ [28]
2-O-Methyl-L-ascorbic acid	1/170 (1/170)	
2-Desoxy-L-ascorbic acid	1/200 (1/70)	
2-Amino-2-desoxy-L-ascorbic acid	1/100; 1/400 (pH 5.5)	1/6 [29]
3-O-Methyl-L-ascorbic acid	1/1500; 1/6000 (pH 5-6); 1/500 (1/50) (pH 8-9)	1/10 - 1/50 [28,30]
5-Desoxy-L-ascorbic acid	1/1500	
Dehydroascorbic acid (methanolate)	10^{-4} (pH 6)	1/2 - 1 [21,31]
2-Keto-L-gulonic acid	1/7000 [b]	$<1/80$ [20,21]
Methyl 2-keto-L-gulonate	10^{-4} [b]	1/5 - 1/7 [20,32]
L-Galactono-γ-lactone	$<10^{-4}$	None detected [33]
L-Gulono-γ-lactone	$<10^{-4}$	None detected [33]
L-Idono-γ-lactone	$<10^{-4}$	

Table VIII
(contd.)

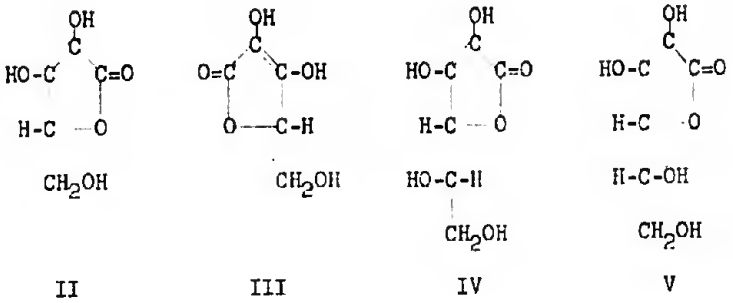
L-Talono-γ-lactone	$<10^{-4}$	
Ascorbigen	1/2000 [b]	<1/20 [34]

[a] With mustard flour in 0.1 M phosphate, pH 6.7.

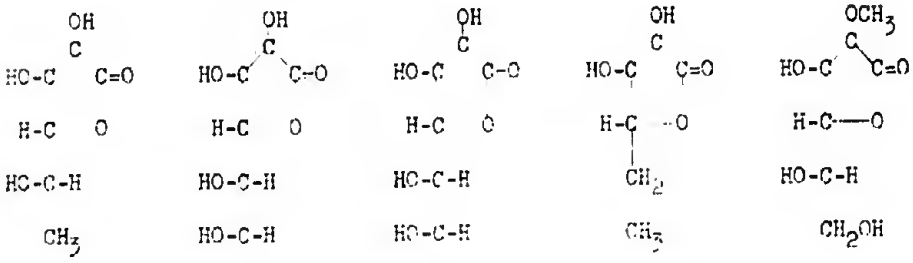
[b] L-Ascorbic acid generated during enzymatic tests.

Determinations on thirty compounds, in large part by Dr. Tom Mabry,[35] with sinalbin and mustard flour in 0.1 M phosphate, pH 6.7, at 20-35° showed no variation greater than a factor of four from results in Table VIII. We signify only the effect of glucoside, already mentioned for flour and D-araboascorbate. The converse influence of the vitamin analogues on the Michaelis constant of sinigrin was only partially examined. D-Glucoascorbate ($2 \cdot 10^{-4}$ M) did not alter the value appreciably from that (10^{-5} M) with enzyme alone. Compounds giving the same limiting velocity as L-ascorbate led, if used at levels producing 2-3% of maximal rate, to Michaelis constants for sinigrin of the same order as when equal speed was caused by L-ascorbate: $2.5 \cdot 10^{-4}$ M with DL-4-ethyl-2-hydroxytetronate, $1\text{-}1.5 \cdot 10^{-4}$ M with L-rhamnoascorbate, D-araboascorbate, L-glucoascorbate, D-erythroascorbate, DL-2-hydroxy-4-isopropyltetronate or 2-O-methyl-L-ascorbate.

The antiscorbutic potencies in Table VIII are complex resultants, now deemed of questionable significance.[36] The match with the coenzymatic properties, though usually better than with susceptibilities to plant or fungal ascorbic oxidases,[37] may be only a curiosity. Anyway, one necessary condition for effectiveness as cofactor of glucosinolase is the same as the Reichstein-Temole criterion[20] for Vitamin C activity of ascorbate analogues, 4-D configuration. The pair of D- and L-erythroascorbic acids (II and III) is the simplest example, and L-ascorbic (IV), D-arabcascorbic (V), 6-desoxy-L-ascorbic (VI), L-rhamnoascorbic (VII) and L-glucoascorbic (VIII) acids offer further illustration. Presumably only the D-component of the racemic 4-alkyl or aryl hydroxytetronic acids is a coenzyme, with twice the efficacy given.



II III IV V



VI VII VIII IX X

Of the compounds tested, several were generously supplied by other laboratories. We are especially indebted to Prof. T. Reichstein for historically unique specimens, including 6-desoxy-L-ascorbic, L-galactoascorbic and L-gulcascorbic acids. The majority of analogues was prepared by Dr. Tom Mabry.[55] Mr. Bennie Badgett synthesized the series of 4-alkyl-2-hydroxytetronic acids. Two undescribed coenzymes of crucial importance deserve particular mention. Ethyl 2-benzoxybutyrate was obtained from ethyl 2-bromobutyrate and sodium benzoate in dimethylformamide and condensed with benzyl benzoxyacetate by metallic potassium according to the method of Micheel and Haarhoff[38] to furnish 4-ethyl-2-hydroxytetronic acid (IX and antipode), m. p. 132-133°. (Anal. Calcd. for $C_6H_8O_4$: C, 50.00; H, 5.59.

Found: C, 50.14; H, 5.70.) 5,6-Isopropylidene-L-ascorbic acid was methylated[39] under nitrogen with dimethyl sulfate in aqueous sodium hydroxide at pH 13-14 to give, after hydrolysis in dilute acid and chromatography on cellulose powder, 2-O-methyl-L-ascorbic acid (X), m. p. 130-131°, $[\alpha]^{27}D$ + 35° (2% in water).[35] (Anal. Calcd. for $C_7H_{10}O_6$: C, 44.21; H, 5.26; CH_3O, 16.31. Found: C, 44.10; H, 5.35; CH_3O, 16.34.)

The essential constancy of the catalytic power of L-ascorbic acid over a pH range of at least four units between the first and second ionizations (pK_1 4.25, pK_2 11.6)[40] demonstrated that the singly charged anion was the effective species. The activities of L-rhamnoascorbic acid, D-araboascorbic acid, D-guloascorbic acid, 2-O-methyl-L-ascorbic acid (pK 3.4)[35] and 2-desoxy-L-ascorbic acid (pK 3.6)[35] were similarly insensitive to pH between 5 and 9. Two compounds, 2-amino-2-desoxy-L-ascorbic acid (pK_a 6.3) and 3-O-methyl-L-ascorbic acid (pK 7.8),[35] ionized near neutrality and showed conspicuous increases of activity (Table VIII) with rising pH.

Nearly all the substances of Table VIII were tested enzymatically with L-ascorbate. No startling synergism or inhibition was uncovered. The rate in presence of two cofactors never exceeded the sum of the rates with the same amounts individually or the maximal speed with Vitamin C. For example, the velocity in 1/6 to 1/4 mg. of L-ascorbate plus 2-1/2 to 5 mg. of 2-O-methyl-L-ascorbate/ml. (cf. Tables IV and VII) was substantially equal to that caused by the L-ascorbate alone, as would be expected if only one enzyme and site were concerned. Compounds with lower limiting speeds, such as 5,6-isopropylidene-L-ascorbate or 3-O-methyl-L-ascorbate, could depress the effect of L-ascorbate, but since no analogue had maximal velocity and

Michaelis constant both small the competitive inhibitions were unspectacular. Substances like D-ascorbate or D-glucoascorbate that were coenzymatically almost inert manifestly did not disturb the system with L-ascorbate.

The inactivity toward glucosinolase of dehydroascorbic acid as the crystalline methanol complex[41] agreed with assays of solutions of L-ascorbic acid freshly treated with partial equivalents of iodine and producing just the effect of unoxidized cofactor. Ascorbic oxidase (cf. Fig. 2) could stop the promoted cleavage of sinigrin with as much as $5 \cdot 10^{-4}$ M vitamin. When a mixture of glucosinolase, sinigrin, L-ascorbate and 2-O-methyl-L-ascorbate was treated with oxidase, the rate of hydrolysis fell to that caused by the methyl ether alone. (Similar experiments were performed with L-ascorbate and 2-desoxy-L-ascorbate or 3-O-methyl-L-ascorbate.) The enzyme activated by Vitamin C and its derivatives was undamaged by the oxidase and the coenzymatic power of 2-O-methyl-L-ascorbate was not attributable to traces of the parent compound.

Products and an Intermediate.--The biochemical degradation of mustard oil glucosides or related compounds need not always furnish isothiocyanates. The most remarkable instance is the recent discovery by Gmelin and Virtanen[42] that certain plants of the mustard family yielding the glucosides (sinigrin, glucotropaeolin) by extraction with methanol give on aqueous maceration organic (allyl, benzyl) thiocyanates: R-S-C≡N, not R-N=C=S. Virtanen has suggested[43] that Lepidium at least contairs an enzyme isomerizing initially formed benzyl isothiocyanate to the thiocyanate. We have made a few preliminary experiments with Thlaspi seed and found that addition of sodium ascorbate did not seem to divert production of allyl thiocyanate. On the other hand, added sinigrin was extensively hydrolyzed to

isothiocyanate, and the observation raised the possibility that the precursor of the thiocyanate was a labile substance not identical with but readily converted into the mustard oil glucoside. The formation of organic thiocyanates offers a profound challenge to be understood but does not appear to be closely connected with the action of Vitamin C.

More clearly relevant to questions here considered are the reports accumulated during the past hundred years of decomposition of mustard oil glucosides to nitriles and sulfur, usually under poorly defined conditions.[44] The two latest enzymatic examples are mentionable. Schwimmer noted[45] that a mixture of myrosin and sinigrin deposited protein at pH 3 but not at 6 and with ascorbic acid produced, only at the lower pH, hydrogen sulfide and a substance giving a color test for nitrile. Gmelin and Virtanen found[46] that 3-indolylmethylglucosinolate and neutral myrosin quantitatively yielded thiocyanate ion as well as indoles, believed to arise from 3-indolylmethyl isothiocyanate, whereas at pH 3-4 sulfur and 3-indoleacetonitrile were also obtained.

Hydrolysis of sinigrin by ascorbate-activated glucosinolase at neutrality gave allyl isothiocyanate, glucose, sulfate and acid. The mustard oil could be determined spectrophotometrically as such or after ether extraction and conversion to allylthiourea.[47] Under mild interference from coenzyme, sulfate was estimated with barium chloranilate[48] and glucose with Sumner's dinitrosalicylate reagent.[6,49,50] All the substances were formed in approximately equivalent amounts and the rate of liberation of glucose, greatly accelerated by vitamin, roughly equalled the titrimetric velocity. After sinigrin (0.05 M) had been completely decomposed by enzyme

(10 μg./ml.) and $6 \cdot 10^{-4}$ M L-ascorbate within half an hour at pH 6 and 15°, the solution proceeded further to mutarotate from + 30° ($[\alpha]^{21}D$, glucose basis) to + 52°.[35] The D-glucose had been formed as the β-anomer and sulfur replaced by oxygen at the 1-position with net retention of configuration.

When sinigrin was hydrolyzed with glucosinolase and ascorbate at pH below 5, a precipitate appeared. At pH 3.5, the lowest used, reaction was incomplete because the enzyme was apparently denatured or carried down. The initial rates of cleavage at pH 3.5-4 were much below optimal, but with sufficient vitamin (0.004-0.009 M) were fifty times faster than without cofactor and presumably corresponded to the enzyme active in neutrality. The precipitate consisted to 70-80% of free sulfur, isolated in yields from decomposed sinigrin that were 8% at pH 4.5, 15-20% at 4 and 50-55% at 3.5. Yields of isothiocyanate were 95% (minimum) at pH 7, 90% at 5, 70% at 4 and 40-45% at 3.5. The ether-soluble products were examined by vapor chromatography on silicone oil and on polyethylene glycol at 70-110° and besides the mustard oil only allyl cyanide was observed. Without care for quantitative recovery, the nitrile was not detected at pH 6 and relative to isothiocyanate constituted some 5% of the analyzed mixture formed at pH 5, 20-40% at 4 and 55% at 3.5.

Like Schwimmer,[45] we noticed evolution of hydrogen sulfide from sinigrin, enzyme and ascorbate below pH 5. However, most of the thioglucoside sulfur was accounted as the element or isothiocyanate and yields at fixed acidity did not seem dependent on vitamin concentration. Reduction of glucone or nascent sulfur appeared quantitatively unimportant.

The ascorbate-promoted enzymatic hydrolysis of sinigrin and probably of other mustard oil glucosides should be conducted at pH above 5 to obtain nearly perfect conversion to isothiocyanate. In more acid medium, the proportion of material following a path to nitrile and sulfur increases and the routes for sinigrin become competitively equal at pH near 3.6. The crude data indicate that the ratio of nitrile or sulfur to isothiocyanate is proportional to hydrogen ion concentration.

Spectroscopic changes during reaction provided insight. Sinigrin had considerably higher molecular extinction in water from 210 to 250 mμ than allyl isothiocyanate ($\lambda_{max.}$ 240 mμ, ε 750), but the mustard oil absorbed a bit more at 255 mμ (ε 400 vs. 350) and longer wave lengths (ε 50 at 270 mμ). Under standard conditions of 0.01 M substrate and 0.5-mm. light path, the effects of glucose and sulfate were negligible and the net result at 255 mμ (or greater) of complete enzymatic hydrolysis in 0.1 M phosphate, pH 6.7, would be a slight gain of 0.02 in absorbance if the products were as expected and the cofactor was unchanged. The anticipation was fulfilled, but at high velocities a transient rise in absorption manifested an unstable intermediate. A drastic example was a reaction at 20° with 0.0015 M L-ascorbate and 40 μg. of enzyme/ml. The initial absorbancy at 255 mμ (ε of vitamin 11500) was summed as 1.05. On first observation, after 40 seconds, the value was 1.635 and rose in another half minute to 1.69, then diminished to 1.075 after ten minutes total, when cleavage was complete.

The absorption caused by intermediate (greatest in example 0.63) became smaller above 255 mμ, decreasing by a factor of ten at 275 mμ and being undetectable at 290 mμ. A temporary fall of extinction was never observed.

The comparative invariance where ascorbate absorbed ($\lambda_{max.}$ 265 mμ, ϵ 15000; ϵ 12000 and 3000 at 275 and 290 mμ) suggested that the cofactor was stationary in concentration and furnished no part of another perceptible compound. The difference spectrum of the intermediate (actually, between it and the isothiocyanate supplanted), roughly disentangled from the effect below 255 mμ of the overall reaction, had the peak at 245-250 mμ.

In duplicate hydrolyses, the absorption at 255 mμ of the maximal amount of intermediate could be measured and at 228 mμ the concurrent rate of cleavage of sinigrin, momentarily without interference from intermediate. With temperature fixed, the observables were proportional: their ratio varied randomly by a factor of 1.3 while the concentrations of enzyme and ascorbate were changed independently by 10 and 12.5 and the velocity covered a range of five fold. Furthermore, the quotient of largest transient absorption and the speed was the same within 20% in acetate buffer, pH 5, or carbonate, pH 9.4, as in neutral phosphate. The steady-state condition implied a mechanism whereby the intermediate, formed from sinigrin by the enzymatic process, decomposed in a first-order reaction not involving enzyme, cofactor or other ions present.

The half-life of the intermediate could be estimated from the rate of appearance of transient absorption at the start, on the assumption that the speed of sinigrin hydrolysis was meanwhile constant, or the fall after the reaction was stopped by ascorbic oxidase. Observation of either change commenced after 30-35 seconds. The half-life found at 20° was 15-25 seconds from the rise and 30-40 seconds for decay. The discrepancy may be real and show yet unravelled complexity, but we suppose that the results bracket the true value and take 25-30 seconds for discussion, corresponding

to a first-order rate constant of 1.5/min. The error is unlikely to exceed a factor of two. Generation of one mole of intermediate for each of sinigrin consumed is the most plausible stoichiometry. We may then calculate for the intermediate, using the average ratio of greatest differential absorption to the velocity, log ε 3.9 at 255 mμ and 4.0 (maximal) at 245-250 mμ. In the specific example given earlier, for which additionally the rate 1 to 1-1/4 minutes from start was measured at 220 mμ as 2.85 μequiv./ml./min., the top concentration of intermediate was 0.0015-0.002 M. The value determined directly as soon explained was 0.0015 M, in satisfactory agreement.

The half-life and concentration of intermediate proved that it could not contain ascorbate. If the cofactor of the example was reduced to 20 ug./ml., the steady-state velocity and quantity of intermediate fell only by a factor of three. Therefore, $1.1 \cdot 10^{-4}$ M ascorbate could yield $5 \cdot 10^{-4}$ M intermediate. In other words, a continuing turnover of $9 \cdot 10^{-4}$ M/min. with $1.1 \cdot 10^{-4}$ M coenzyme required that any transient compound incorporating one mole of ascorbate per substrate reacted and breaking down by a first-order reaction possess a half-life less than 6 seconds.

When a solution containing much intermediate was swiftly acidified to pH 1-3, the absorbance at 255 or 260 mμ, first observed 40-55 seconds later, thereafter changed little (less than 0.06; 0.5-mm. path). The spectrum of the mixture after five minutes showed, minus effects of ascorbic acid, sinigrin and mustard oil, general absorption of 0.15-0.25 from 300 to 250 mμ. The intermediate seemed to decompose faster in acid than neutral medium. The acid solutions became cloudy and formed a precipitate within a

few minutes. No such turbidity appeared at pH 2 when sinigrin or ascorbate were omitted or the enzyme concentration was initially small and was raised after acidification. The solid consisted to at least half of elemental sulfur, isolated (3 to 3-1/2 mg.) after recrystallization in 40-50% yield from the calculated quantity of intermediate. When the solution of the spectroscopic example was brought to pH 2 after 75 seconds and isothiocyanate (0.002 M) and remaining sinigrin were determined, the deficit from original substrate was 0.0015 M. If 1 µg. of enzyme/ml. was used during 30 minutes, the same amount of sinigrin was hydrolyzed but was all recovered after acidification as mustard oil. (The extra enzyme, to consume the missing material or be part of the intermediate, would have the equivalent weight of 30. The yield of isothiocyanate on complete hydrolysis was normal with much glucosinolase.) Hence the concentration of intermediate was verified and its non-enzymatic decomposition was shown to control formation of sulfur and presumably nitrile in acid or of isothiocyanate near neutrality.

The ascorbate-activated enzymatic hydrolysis of sinigrin was also observed spectrophotometrically in acetate buffer, pH 5, in the presence of 0.01 M zinc or cadmium ions, which did not react with substrate or coenzyme or alter the final products. The zinc depressed the hydrolytic rate by 15% and the transient absorption by a similar slight amount. Cadmium, however, caused both a 40% drop in velocity and a 70% rise in maximal absorption by unstable intermediates, so that the ratio of the second quantity to the first increased over its previously constant value by a factor of nearly three. The change of absorption at 255 mµ with time indicated that the half-life of the intermediates had at least doubled.

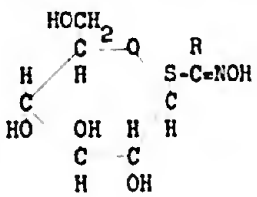
Transient absorption at 255 mu was observed by Schwimmer[9] during reaction of sinigrin and myrosin without ascorbate. Using 250 µg. of enzyme/ml., he obtained a hydrolytic velocity of roughly 0.05 µequiv./ml./min., smaller by a factor of fifty than in our example previously cited. It is notable that the passing absorbance, on the order of 0.2 with 1 cm. path, was also reduced in approximately the same ratio, as would correspond to involvement of the same intermediate regardless of Vitamin C. Schwimmer interpreted the changes at 255 mu in terms of "the formation of side reactions or of relatively stable intermediates" or "the spectrum of the enzyme-substrate complex."

DISCUSSION

Enzymes that liberate isothiocyanates from glucosides are found in dicotyledonous flowering plants that yield such substances,[51] fungi,[49,52] and bacteria, including those of the human gastrointestinal tract.[53] Fungal sinigrinase,[49] according to our experiments, is not affected by L-ascorbate or its oxidase. Therefore no absolute requirement exists in the cleavage of glucosinolates for Vitamin C.

The evidence is that yellow mustard seed contains at least two enzymes that catalyze the same reaction, hydrolysis of mustard oil glucosides. One enzyme, corresponding to the classical myrosin, acts equally in presence or absence of ascorbate. The other enzyme needs Vitamin C as cofactor and is the proper ascorbate-activated glucosinolase. The enzymes have not to our knowledge been completely separated and they possess similarities, including stability to heat and breadth of pH optima. However, myrosin activity cannot well be ascribed to traces of ascorbate or to the apo-enzyme of ascorbate-activated glucosinolase. Although the rate enhancement by optimal cofactor versus none in glucoside cleavage varies for mustard extracts by fifty fold, the reaction with substrate only, whether of whole flour or purified glucosinolase (cf. Fig. 2), is unaffected by ascorbic oxidase. Likewise, the activity of the enzyme preparations against substrate alone persists after dialysis. The relation of velocity to ascorbate concentration (Table IV) is consistent with activation of only one enzyme. Finally, the apparent discontinuity between Michaelis constants of sinigrin observed without cofactor or in the promoted hydrolysis with little ascorbate suggests that the two reactions and enzymes are best treated as independent.

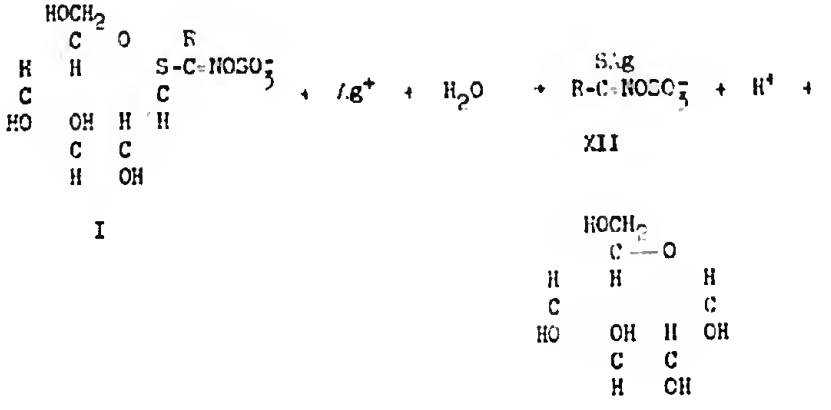
The report[49,54] that desulfoglucotropaeolin (S-β-D-1-glucopyranosyl-phenylacetothiohydroximic acid; XI, R = $C_6H_5CH_2$) was attacked very slowly if at all by mustard enzyme antedated knowledge of the role of Vitamin C. Experiments by Dr. George Dateo[7] have clarified the substrate requirements. Desulfoglucocapparin (XI, R = CH_3) at 0.008 M was hydrolyzed to glucose by our glucosinolase without cofactor at roughly one thirtieth of the rate for sinigrin likewise, and 0.015 M 2,4-dinitrophenyl β-D-1-thioglucopyranoside was cleaved nearly as fast as sinigrin. However, the reactions of the uncharged compounds were hardly affected by ascorbate. The results suggested an ascorbate-independent general thioglucosidase, in accord with previous observations that myrosin hydrolyzed desulfosinigrin[55] (XI, R = $H_2C{=}CHCH_2$) and the dinitrophenyl thioglucoside,[56] but did not settle that only one enzyme was involved and was myrosin. The ascorbate-activated glucosinolase was highly specific among substrates to mustard oil glucosides.

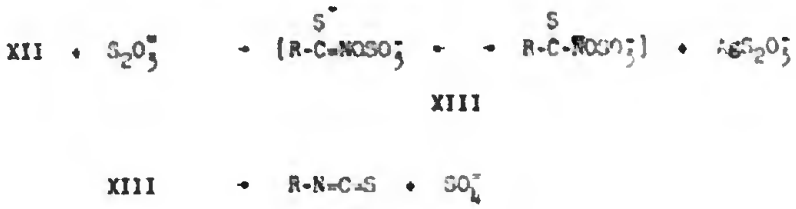


XI

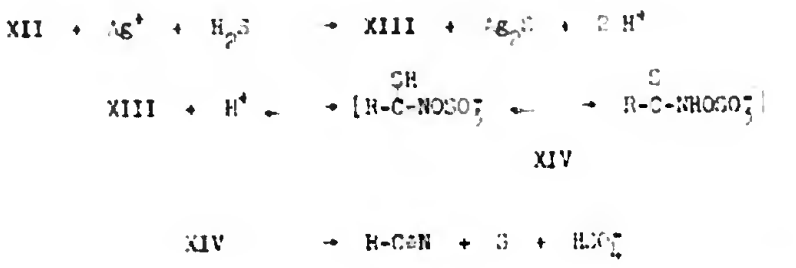
The first consideration in the mechanism of vitamin-promoted enzymatic hydrolysis of glucosinolates is the detectable intermediate. The substance is a fragment of the glucoside and decomposes spontaneously to nitrile and sulfur in acid, isothiocyanate in neutral solution. One sort of compound with such general properties is a thiohydroxamic acid. For example, phenylacetothiohydroxamic acid[54,57] decomposes at room temperature in hydroxylic

solvents to benzyl cyanide and sulfur, whereas the solid sodium salt furnishes N,N'-dibenzylthiourea, evidently through Lossen rearrangement to alkali and isothiocyanate. However, phenylacetothiohydroxamic acid is isolable, can be purified by extraction from ether with cold aqueous carbonate and acidification,[57,58] and seems to represent a more stable class than the intermediate. A closer and apparently satisfactory match is given by the aglucones or thiohydroxamic acid-O-sulfonates. The aglucones are known as their silver salts[4,55,59] (XII), obtained with α-glucose[60] and acid from the glucosides and silver nitrate. The silver atom depicted is covalently bound as a mercaptide and another atom is present in the isolated salts as cation, either silver or its diammine. The salts, including silver sinigrate (XII, R = $H_2C{=}CHCH_2$), furnish isothiocyanates on treatment with thiosulfate or warm chloride but sulfur and nitriles with hydrogen sulfide or hydrochloric acid. Thiosulfate is a nucleophile and must attack silver with displacement of thiohydroxamate-O-sulfonate (XIII), which undergoes Lossen





rearrangement.[7] Hydrogen sulfide likewise captures the silver of (XII) but liberates hydrogen ion so that decomposition proceeds as from the thiohydroxamic acid-O-sulfonate (XIV).



The breakdown of (XIV) has formal analogies, including fission of ketoximes to nitriles by Beckmann transformations of the second kind.[61] Only the presence of acid sets off the path to nitrile and sulfur from the route to isothiocyanate. The reactions are rapid and complete, and no evidence supports suggestions that nitrile formation proceeds through hydrolysis of (XIV) to the thiohydroxamic acid[44] or that (XIV) could survive.[45] We identify the newfound transient in the ascorbate-promoted enzymatic hydrolysis of sinigrin with the sinigrate or vinylacetothiohydroxamate-O-sulfonate ion (XIII, R = $H_2C{=}CHCH_2$).

Whether the properties of the enzymatic intermediate are expected for sinigrate must be considered. The ultraviolet absorption maximum accords with that of phenylacetothiohydroxamate in alkaline methanol at 247 mμ

(log ϵ ca. 3.8).[58] The autonomous first-order decomposition near neutrality fits a Lossen rearrangement. The half-life is smaller than that of acetohydroxamate-O-sulfonate (XV), which splits to sulfate and methyl isocyanate (eventually N,N'-dimethylurea),[7] by a factor of 50-100. Since the rate of decomposition of the enzymatic

$$\underset{\text{XVI}}{CH_3\overset{O}{\overset{\|}{C}}NHOSO_3^-} + H^+ \longrightarrow \left(CH_3\overset{O}{\overset{\|}{C}}\text{-}NOSO_3^- \leftrightarrow \underset{\text{XV}}{CH_3C{=}NOSO_3^-}\right)$$

$$\text{XV} \longrightarrow SO_4^{=} + CH_3NCO \xrightarrow{H_2O} CH_3NHCONHCH_3$$

intermediate is constant from pH 9 to 5 and appears faster at pH 1-3, yet at 3.5 some 40% of the product comes from the same reaction as at neutrality, more than that fraction of intermediate is in the same state and the pK_a of any protonated form is less than 3.5. The equilibrium of (XIV, R = $H_2C{=}CHCH_2$) and (XIII) may be estimated from that[7] of acetohydroxamic acid-O-sulfonate (XVI), pK_a 8.0, and (XV). The vinyl group should reduce the pK by 0.4 or so,[62] but the major effect comes from the divalent sulfur atom. The palpable acidity of thioanilides in contrast to anilides was known of old,[63] and in isopropanol a recent study[64] has shown thioacetamide to be a stronger acid than acetamide by a factor decidedly more than ten thousand. Hence it is plausible to assign vinylacetothiohydroxamic acid-O-sulfonate an effective pK_a near 3. The great acidity of the aglucone hydrogen atom that is supplanted is a noteworthy peculiarity of mustard oil glucosides.

The existence of (XII) raised the possibility that the enzymatic intermediate could be trapped as a metal complex. However, since the metal would preferably not combine so avidly with sulfur as to react with sinigrin

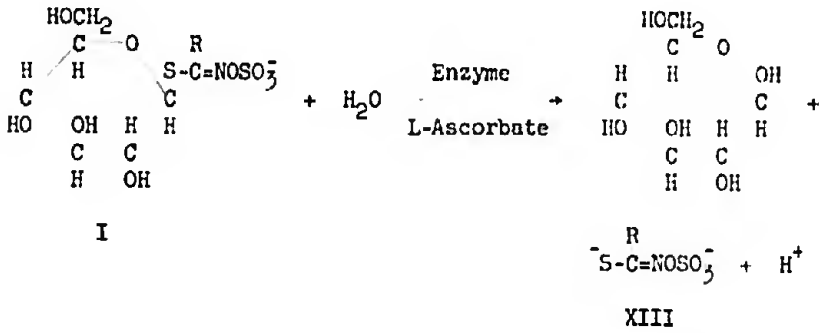
or isothiocyanate or to denature the enzyme, the complex would be less stable than the silver salt. It is also desirable that the metal ion should not form an insoluble sulfate or be reduced by ascorbate, and that it should be transparent to ultraviolet light and soluble at pH of at least 5. Zinc did not seem to affect the intermediate, but cadmium, which is said[65] to have greater affinity for thiol groups, did give clear evidence of formation of a longer-lived species.

Further investigation of (XIV) and its derivatives would be feasible and instructive. For example, if our view is correct, decomposition of silver sinigrate with suitable nucleophiles (bromide, iodide) in acidic buffers should yield the same dependence of product ratios on pH as from the ascorbate-promoted enzymatic hydrolysis of sinigrin. Spectroscopic detection of (XIV) from reaction of (XII) and neutral hydrogen cyanide might be possible. The like tests of light absorption[9] and product determination[46] could be applied more critically than before to elucidate the paths of vitamin-independent enzymatic cleavages of mustard oil glucosides.

An ambiguity about the formulation of (XIII) is whether the ion may exist in two stereoisomeric forms, interconverted doubtless with ease but differing in configuration about the carbon-nitrogen partial double bond and presumably in spectra and modes of decomposition. Attempts to obtain syn-anti stereoisomers of the glucosides (I) have not yet succeeded.[7] Since intermediates that readily stereomutate may intervene generally in conversions of glucosinolates to mustard oils, the previous[5] assignment of relations about the carbon-nitrogen double bond in (I) from the migration was unjustified.[58] It is hoped that X-ray studies[66] of sinigrin and the

isomorphous ammonium and thallous myronate monohydrates[58] indicating syn-configuration of the sulfur atoms will attain completion.

By the present interpretation, the reaction that directly involves enzyme and ascorbate with sinigrin is hydrolysis of the glucosyl-sulfur bond. The enzyme is a specific, ascorbate-requiring thioglucosidase. The general view of myrosin as primarily a thioglucosidase was indicated as soon as the structure of the substrates was known[5] and was elaborated theoretically by Lundeen[58] and others.[6,44,49,55] For the ascorbate-catalyzed process, we have now observed the



I XIII

stepwise character and shown that the spontaneous Lossen rearrangement is faster than any enzymatic transformation of the intermediate under our conditions. The earlier suggestion[54] that glucosyl and sulfate groups were lost simultaneously was needless. Since desulfoglucocapparin and desulfoglucotropaeolin do not at 0.02 M inhibit the vitamin-activated hydrolysis of sinigrin (0.005 M), the sulfate group appears important for combination of substrate and glucosinolase as well as for electron withdrawal[58] that weakens the thioglucoside link.

The question whether the ascorbate-independent cleavage of mustard oil glucosides results from the combined action of two enzymes, a thioglucosidase and a sulfatase, is under dispute. Nagashima and Uchiyama have argued staunchly from their experiments[6,55,67] that the myrosin of yellow mustard is a single enzyme. On the other hand, Gaines and Goering[50] have claimed that a similar preparation from Oriental mustard (Brassica juncea) can be separated into sulfatase and thioglucosidase, and that the latter enzyme can also hydrolyze certain O-glucosides. Gaines and Goering did not study what intermediates might be produced from mustard oil glucosides by the resolved enzymes. The sulfate group can certainly be hydrolyzed by molluscan sulfatase[55] or mild acid,[7] but the resulting oximes (XI) are essentially inert to further transformation into isothiocyanates. Yet if a thioglucosidase releases glucose but no isothiocyanate or sulfate from sinigrin (during four hours at 37°), the aglucone cannot go free as has been proposed.[44] The successive reactions of sinigrin with silver and thiosulfate ions, taken as a model for a two-stage enzymatic system, show that some bound form of the aglucone must be provided.

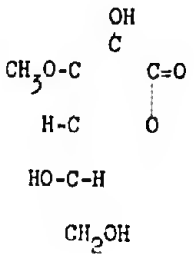
The specific activities of purified myrosin[50,67] are recorded as 7-16 μmoles of sinigrin hydrolyzed/min./mg. protein at 37°, less by a factor of twenty or more than found for the ascorbate-activated enzyme. The vitamin-requiring glucosinolase indeed produces a specific rate of the same magnitude as for crystalline bacterial β-galactosidase[68] with its best substrate.

The role of ascorbate in the glucosinolase reaction has still to be examined. A desirable first remark is that spectroscopic and kinetic evidence indicates no substantial accumulation of any intermediate under

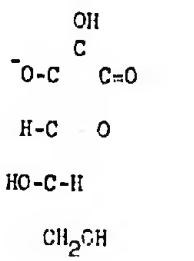
conditions of the data in Tables IV-VII. The presence of (XIII) (maximum $3 \cdot 10^{-4}$ M) cannot disturb the titrimetric results anyway at pH above 4 and no other transitory compound has been detected. The concentrations of cofactor appear essentially unchanged during reaction and the rates pertain to the enzymatic stage.

L-Ascorbate appears to act through rapid, reversible formation of a ternary complex between ascorbate, glucoside and enzyme. The following circumstances are indicative. First, the reaction starts without evident induction period when ascorbate or glucosinolase are added and stops promptly when the coenzyme is selectively destroyed (Fig. 2). Second, the variation of rate at high sinigrin level with concentration of ascorbate (Table IV) suggests a Michaelis-Menten relationship. Third, the existence of eight analogues (cf. Tables V-VII) giving the same limiting velocity as L-ascorbate in 0.01 M sinigrin but with larger Michaelis constants can most simply be attributed to combination with lower affinities for enzyme plus substrate but at the same site to form the identical catalytic atomic configuration. Fourth, the Michaelis constant of D-araboascorbate depends on the glucoside that joins with the enzyme. Fifth, the Michaelis constant of sinigrin varies with concentration of L-ascorbate. The value with little cofactor presumably corresponds roughly to reversible combination of sinigrin and enzyme alone, the other extreme to union of glucoside with the enzyme-ascorbate complex. The increase caused by ascorbate implies the reality of both binary complexes and that either anion bound opposes addition of the other. At low concentrations of any cofactor (II) or (IV)-(X), the Michaelis constant of sinigrin is of the same magnitude.

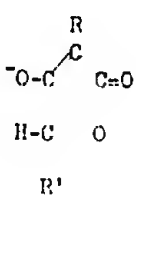
Since the 2-methyl ether (X) of ascorbic acid is 25000 times more acidic than the 3-O-methyl isomer (XVII), it may be inferred that the predominant mono-anion of ascorbic acid is the one (XVIII) formed by dissociation of the 3-hydroxyl group. That (X) and 2-desoxy-L-ascorbic acid are



XVII



XVIII



XIX

considerably more active as coenzymes than (XVII) or the 3-deroxy compound confirms that (XVIII) is the effective form of L-ascorbate. The negative charge in (XVIII) is of course distributed to the 2-carbon and 1-oxygen atoms. The maximal velocity produced by L-ascorbate is approached also with sufficient of the anions of (IX) or (X), and therefore the 2-, 5- and 6-hydroxyl groups of (XVIII) are important for combination with enzyme-substrate but are not catalytic centers. The unit of coenzymatic function appears to be a tetronate ion (XIX) with 2- and 4-substituents that enhance affinity for the enzyme. The requirement for (XIX) is not absolute, since other ions resembling (XVIII) have some effect. Thus, the salt of (XVII) can furnish one tenth of the maximal rate of (XVIII).

The general appellation for ions like (XIX) is enolate or base. No relation between base strength and greatest enzymatic velocity need be expected for (XIX) and analogues because steric changes near the catalytic center can

be major and the relative basicity of cofactors attached to enzyme is unknown. The idea that the coenzyme might act as a base or nucleophile is however supported by the character of the reaction, displacement of a strongly electron-attracting group from a 1-glucosyl residue. The ion (XIII) liberated is normally less basic than ascorbate. No evidence exists of a glucosyl-enzyme or glucosyl ascorbate intermediate, but the stereochemistry accords with prediction.[58] Whereas reaction of sinigrin and silver ion involves electrophilic displacement on sulfur and nucleophilic displacement by water at the 1-carbon atom with inversion, the net retention of configuration in glucosinolase cleavage suggests two nucleophilic displacements at the 1-position,[69] first by enzyme-ascorbate and then by water. (The statement in a recent review[70] that myrosin produces α-glucose from sinigrin is misunderstood. The isolation[71] of α-glucose from a solution that had been heated repeatedly and evaporated bears no relation to the original composition and simply reflects that this anomer is the ordinary crystalline form.) We may summarize with the hypothesis that ascorbate behaves toward the thioglucosidase as a reversibly dissociable base, which is closely connected with the active nucleophilic group.

The foregoing results are entirely apart from current interest in ascorbate because of its physiological functions in mammals and its chemical behavior as a reducing agent and source of free radicals.[72] Any general significance of the study here presented might be taken in two ways. Practically, the employment of compounds like (IX) and (X) to discriminate the essential groups of ascorbate might be helpful in future investigations. Theoretically, the view of the ascorbate ion (XVIII) as a base stable over

a wide pH range surrounding neutrality can be augmented by recognition of the simultaneous presence of the weakly acidic 2-hydroxyl group. Since the aglucones of glucosinolates are strongly electron-demanding and the rearrangement is a step to itself, reason exists why the acid group of ascorbate is unneeded during the hydrolysis of (I). In other reactions, however, the presence of both acidic and basic centers in proximity may confer catalytic powers, just as the reactivity of phenolate ion toward acyl halides is uniquely enhanced by an o-hydroxyl group.[75] For both catechol and ascorbic acid, the existence of oxidation-reduction equilibria should not dominate thought to the exclusion of possible functions as acid-base catalysts.

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